Postnatal Vitamin D Supplementation Normalizes Neonatal Bone Mass Following Maternal Dietary Vitamin D Deficiency in the Guinea Pig

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Abstract

Since vitamin D deficiency is common at birth, the objective of this study was to test if postnatal vitamin D supplementation would normalize bone mineralization. Forty guinea pigs were randomized to receive a diet with or without vitamin D_3 during pregnancy. Newborn pups were randomized to receive 10 IU of vitamin D_3 or a placebo daily until d28. Measurements at birth and d28 included whole body and regional bone mass, osteocalcin and deoxypyridinoline, plus biomechanical testing of excised tibias and femurs. Offspring from deficient sows had lower body weight, whole body and tibia bone mineral content (BMC) and lower osteocalcin and biomechanical integrity. By d28 this group had lower whole body bone density and femur BMC, unless supplemented. Interactions with gender showed males continued to have low 25(OH)D despite supplementation. Therefore, neonates born to sows with dietary vitamin D deficiency require supplemental vitamin D to support normal bone mineral accretion.

Résumé

Puisque un déficit de vitamine D est commun à la naissance, l'objectif de cette étude était d'examiner si un supplément de vitamine D post-natale normalisera la minéralisation d'os. Quarante cobayes ont été randomisés pour recevoir un régime avec ou sans vitamine D3 pendant la grossesse. Des chiots nouveau-nés ont été randomisés pour recevoir 10 IU de vitamine D3 ou un placebo quotidien jusqu' à jour 28. Les mesures à la naissance et au jour 28 ont inclus la masse d'os régionale et du corps entier, l'ostéocalcine et la deoxypyridinoline, plus l'essai biomécanique des tibias et fémurs excisés. Les chiots des mères insuffisants ont moins du poids corporel, masse osseuse du corps entier et du tibia ainsi que, d'osteocalcine et d'intégrité biomécanique en comparaison au groupe suffisant. A jour 28, ce groupe avait une densité d'os du corps entier et de fémur plus bas s'ils n'étaient pas supplémenter. Les interactions avec sexe ont montré que les mâles continué à avoir un niveau bas de 25(OH)D malgré la supplément. Donc, nouveau-nés, nés des mères avec l'insuffisance diététique de vitamine D ont besoin de la vitamine D supplémentaire pour soutenir leur accroissement normale des minéraux osseuse.

Abbreviations

Bone Mineral Content	BMC
Bone Mineral Density	BMD
Deoxypyridinoline	DPD
Dual-energy X-ray absorptiometry	DXA
Vitamin D-binding Protein	DBP
Vitamin D Receptor Protein	VDR
Ultraviolet B	UVB

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1.0 Chapter One: Literature Review

1.1 Physiology of Vitamin D

1.1.1 Function of Vitamin D

Vitamin D is essential to health and can be acquired through endogenous synthesis or exogenous sources (1). Regardless of the source, the active form of vitamin D ($1,25(OH)_2D$) is involved in regulating calcium and phosphorous concentrations in the blood by promoting their absorption from food in the small intestine and by promoting re-absorption of calcium from the kidneys. Without vitamin D humans would only be able to absorb 10-15% of dietary calcium and approximately 60% of phosphorous through passive transport. In the presence of vitamin D however, the efficiency of calcium and phosphorous intake is increased about 30-40% and 80%, respectively, through active transport processes (1). This supports bone formation and mineralization and is required to develop and maintain optimal peak bone mass.

1.1.2 Vitamin D Acquisition

Vitamin D can be acquired in the diet (typically from milk and margarine in Canada) or through endogenous synthesis, which is triggered by ultraviolet beta (UVB) radiation from the sun (1-3). There are two dietary sterols that can form precursors for vitamin D. One form is found in plants and is called ergosterol that can be activated through irradiation to form ergocalciferol (also known as vitamin D_2 or ercalciol). This compound is most commonly sold commercially to prevent or treat rickets

and used in some plant beverages such as rice beverage. The other sterol, 7-dehydroxycholesterol, is mammalian derived typically from sources such as fish or liver (see **Table 1-1** for selected dietary sources of vitamin D). It is found throughout both the dermis and epidermis and when it is exposed to a specific wavelength of UVB light (290-315 nm) it forms previtamin D_3 (precalciferol). Within two to three days precalciferol is thermolised to cholecalciferol (vitamin D_3) and will diffuse from the skin to the blood stream where is it transported to the liver by α-2globulin vitamin D-binding protein (1).

Factors that can affect the endogenous synthesis of vitamin D include:

- Skin pigmentation: melanin competes for UVB photons. Therefore the more melanin in the skin, the less UVB photons are available for endogenous synthesis of vitamin D (4).
- Wearing sunscreen: an SPF greater than 8 eliminates endogenous synthesis (3).
- The time of year: for example in Canada UVB radiation does not adequately reach latitude of 42 ° N between October to March/ April to elicit endogenous synthesis of vitamin D. Thus during this time there is increased risk for vitamin D deficiency. This is in part due to the reduced UVB and is attributed to the zenith angle, which is in turn

related to the shortening of days during the winter months (5).

- Clothing: Vitamin D synthesis cannot occur if the sunlight passes through clothing or windows (3).

The factors listed above are important to consider since vitamin D deficiencies have been detected in pregnant women and children world wide due to cultural or religious clothing habits (3), skin pigmentation (4) and also due to an increase in sedentary lifestyles that is associated with less exposure to UVB (2,6). In addition to reduced endogenous synthesis of vitamin D, dietary intake of vitamin D is felt to be inadequate relative to current recommendations and based on low vitamin D status year round (4).

Table 1-1: Selected Foods Containing Vitamin D

Food	International Units (IU) per serving*
Cod liver oil, 1 Tablespoon	1279
Salmon, cooked, 100 g	272
Mackerel, cooked, 100 g	104
Tuna fish, canned in oil, 165 g	46
Milk, non-fat, reduced fat, and whole, vitamin D fortified, 1 cup	107
Margarine, fortified, 1 Tablespoon	75
Egg, 1 whole (vitamin D is found in egg yolk)	22
Liver, beef, cooked, 75 g	21
Breast milk, human, whole, mature, 1 Litre	41.6
Breast milk, human (1 Litre)	~10-30**

* Health Canada, Canadian Nutrient File, 2007

** Ziegler et al (7), mature milk not specified.

Regardless of the source of vitamin D, both dietary and endogenously acquired forms are only biologically active following further metabolism through hydroxylation. The first hydroxylation occurs in the liver at the 25^{th} carbon and the subsequent hydroxylation occurs in the kidney at the 1^{st} carbon, it is now the metabolically active form $1,25(OH)_2D$ (8). The circulating serum concentration of 25(OH)D is commonly used to indicate vitamin D status as it reflects both dietary and endogenous sources. Since the half-life of 25(OH)D is 2-3 weeks it is considered to be the most reliable measure of a person's cumulative vitamin D status (11). The half-life of the active form of vitamin D, $1,25(OH)_2D_3$, is only 4-6 hours making it a difficult and unrealistic measure of vitamin D status (11).

1.1.3 Functions of Vitamin D at the Cellular Level

Vitamin D status continues to be a public health concern due to its role in the prevention of rickets, osteomalacia and osteoporosis. It has been suggested that the role vitamin D plays may not be limited to the regulation of calcium in the body. It may help maintain a healthy immune system and help regulate cell growth and differentiation (9). The discovery of nuclear vitamin D receptor proteins (VDR) in over 30 tissues throughout the body including bone, kidneys, lungs pancreas, brain and muscle has lead to this theory. Vitamin D is also proposed as a factor in the prevention of various diseases such as cancer, diabetes and multiple

sclerosis (8-11). However its main function is to maintain adequate blood calcium levels. This is done through interacting with receptors in the small intestine. One, twenty five dihydroxyvitamin D enhances absorption of intestinal calcium and dietary phosphorous by increasing the efficiency of their absorption (12). If there is inadequate dietary calcium available to meet requirements $1,25(OH)_2D$ and parathyroid hormone mobilizes monocytic stem cells in the bone marrow to become mature osteoclasts. Interaction with the VDR in osteoblasts ultimately leads to the dissolving of the bone mineral matrix in order to restore blood calcium, signal transduction and neuromuscular activity (12).When calcium status is adequate, vitamin D also stimulates bone formation by enhancing bone matrix synthesis and mineralisation (13).

1.1.4 Interpreting Vitamin D Status

The scientific counsel recommendations for vitamin D intake were developed by the Institute of Medicine (IOM) of the National Academy of Sciences. The Dietary Reference Intake (DRI) value for vitamin D is currently an adequate intake (AI) value set at 200 IU/d from birth to 50 years of age with higher values thereafter (14).

Most health professionals define vitamin D deficiency as a serum 25(OH)D level of less then 50 nmol/L however 20 to 37.5 nmol/L is considered the lower limit for the normal range (1,15). In infants a vitamin D deficiency is defined as a serum 25(OH)D level less than 27.5 nmol/L

(15). A serum 25(OH)D level greater than 75-100 nmol/L is preferred in adolescents and adults since vitamin D status is inversely related to parathyroid hormone levels until this level is reached (1).

Vitamin D deficiency is a problem in Canada and other countries where seasonal sun patterns affect inhabitants ability to endogenously synthesize vitamin D. Countries closer to the equator also report vitamin D deficiency within their populations that is thought to be due to an increase in sedentary lifestyle as well as the religious or cultural requirement to remain fully covered (16-19). Vitamin D deficiency is an issue throughout the lifespan. In infants it can lead to rickets which is characterized by abnormal growth of bones resulting in bowed legs, outward-bowed chest and rachitic rosary on the ribs (20). In adults, it manifests as osteomalacia with the symptoms include bending of the spine and bowing of the legs. It can also lead to muscle weakness and bone pain, which can go unnoticed during the initial stages of the deficiency. Long term vitamin D deficiency, even that which does not lead to rickets or osteomalacia can over time play a role in adult bone loss (21). This reduction in bone mass in older persons is known as osteoporosis and is characterized as bones becoming porous and fragile (21).

Vitamin D toxicity is rare and is most likely linked to high intakes of supplements or cod liver oil. It is unlikely to occur from intake of food or sun exposure. The symptoms of vitamin D toxicity include nausea, vomiting, poor appetite, constipation, weakness, weight loss and elevated

blood concentrations of calcium that cause mental confusion (12). High blood concentrations of calcium also can cause heart rhythm abnormalities and eventually calcification of soft tissues such as the renal tubules of kidney (14). The tolerable upper limit for vitamin D is currently 1000 IU for infants from birth to 12 months of age and 2000 IU thereafter (14).

1.2 Fetal programming

There is some evidence that the intrauterine environment does play a role in later bone health. Evidence is growing for the theory of fetal programming which suggests that the future growth and development of the skeleton is influenced by adverse effects in the critical period *in utero* where the greatest amount of growth and development take place (22,23). This suggests that what happens *in utero* has a significant effect on the risk of developing chronic diseases as well as future quality of life. Fetal programming may influence susceptibility to diseases such as cancer, cardiovascular disease, hypertension, diabetes and obesity (23). In relation to bone health and vitamin D status, epidemiological studies such as the one by Arden et al. (24) have shown that adult women born small for gestational age have higher circulating $1,25(OH)_2D$ (24). Thus the *intra uterine* environment may affect adult bone mineral density (BMD).

Data now suggest that environmental factors, nutrition included, as early as in fetal life have longstanding effects on bone mass. Limited *intra uterine* nutriture (i.e. poor placental development leading to growth

restriction) is related to lower bone mass in children (25) and elderly men (26) plus a higher incidence of hip fracture in men and women (27). Evidence indicates that both growth restricted term born infants (low birth weight <2500 g) as well as prematurely born infants have longstanding high bone turnover causing osteopenia (28,29). Low birth weight is an indicator of nutritional status *in utero* and it is linked to a significant (0.86%) increased rate of mortality by age 75 in both men and women (30). These individuals are at higher risk of suboptimal peak bone mass and long term risk of poor bone health problems including osteopenia compounded by osteoporosis and fracture (32). Weight at one year has also been shown to have a significant effect on bone mineral content (BMC). In a longitudinal study, a higher weight at 1 year of age was shown to be associated with increased BMC at the lumbar spine and femoral neck at 27 years of age (31).

In addition, laboratory studies using rats have shown that the offspring of pregnant rats deficient in vitamin D have abnormal skeletal growth and morphology despite provision of a supplement to the dam during lactation (32,33). It is possible that if the supplement had been given directly to the pups instead of through mothers' milk the supplement would have yielded better recovery. Alternatively, it is possible that there is a lifelong negative effect of vitamin D deficiency *in utero* on subsequent bone mass.

Experimentally controlled studies in animals support these

descriptive human studies. For example, the small sized rat pup at birth that is fed a regular diet thereafter, has compromised bone mass in adulthood (22). Such observation suggests that bone mass does not fully recover after early life insult despite optimal nutrition and that bone mass can be programmed by early life nutritional insult.

In contrast, better nourishment as indicated by higher yet normal birth weight (34) is associated with higher adult whole body, lumbar and hip bone mass. This association holds true even after accounting for age, sex and height plus adult behavioural factors including smoking, alcohol, dietary calcium and exercise (34). A number of factors are known to affect human infant bone mass at birth including vitamin D status of mother and infant (35), maternal calcium intake (36), activity (37), and smoking (37) while pregnant. At birth, infants with higher vitamin D status have higher weight adjusted bone mass (35). Longitudinal studies reveal that maternal intake of fat, phosphorous (38) and smoking (39) while pregnant are linked with the child's subsequent reduced bone mass at 8 years of age. In one study, maternal consumption of milk while pregnant accounted for 35% of the variation in femoral neck BMD in the children at 8 years of age (38). This study was conducted in Australia where milk is not fortified with vitamin D. The authors did not indicate if the milk was full fat or otherwise and thus the contribution of naturally occurring vitamin D to this association is not clear. However, maternal vitamin D status in pregnancy and thus fetal exposure *in utero* is related to

bone mass in children (40). Of those studied by Javaid et al. (40) 31% of the mothers had low circulating levels of vitamin D during late pregnancy. This resulted in lower whole body and lumbar spine BMD in their children at the age of 9.

Not only does the mother's status during pregnancy play a role in the bone development of the child, but the child's status during infancy will play a role as well. Infants who are vitamin D deficient with a deficiency being defined as less then 27.5 nmol per litre 25(OH)D had an 8.5% reduction in their whole body BMC when it was corrected for body weight. Of the infants studied 36% were defined as vitamin D deficient; 46% of their mothers were also reported to be deficient as well (35). These same infants were studied at 6 months of age to determine if the season in which they were born had an effect on their bone development. As mentioned earlier season can affect vitamin D synthesis. It was found that the vitamin D status of the infants varied by season and when tracked showed changes in bone mineral accretion of the lumbar spine by 6 months of age. The infants born in the winter and spring had lower bone mineral accretion than the infants born in the summer or fall (41). This is significant since those with lower accretion are at an increased risk for spinal fracture later in life.

In a randomized controlled trial of vitamin D supplementation of breast-fed infants compared to placebo, radial BMC was transiently elevated after 3 months of supplementation when compared to infants who

did not receive a 400 IU/day supplement of vitamin D (42). In a prospective cohort study, providing a vitamin D supplement in infancy (median duration of 12 months) had a longstanding association with higher BMD at 7 to 9 years of age (43). The distal radius was 6% higher and the femoral neck 9% higher in the girls who received a vitamin D supplement in infancy (43). These are common sites for osteoporotic fracture later in life (44). Thus there is some evidence that maternal vitamin D status and higher intake of foods containing vitamin D is associated with higher bone mass in infants and subsequently in childhood.

Conversely, if vitamin D deficiency is not corrected, the consequences are not clear. It is also not clear how much vitamin D is required in infancy for optimal outcomes. Longitudinal studies that link vitamin D status at birth, subsequent dietary vitamin D intake from foods and supplements with peak bone mass and maintenance with aging are not available. The most realistic area to target is to improve vitamin D status of the mother to prevent deficiency from the outset. Yet, it seems that there is not enough evidence to suggest that maternal deficiency of vitamin D has any longstanding consequences to the offspring's bone health. Human studies both descriptive and randomised clinical trials might be compromised by the issue of confounding variables. Nonetheless, similar results are observed in animal studies where these variables are highly controlled.

The mechanism in which young animals acquire vitamin D was investigated using the rat model of intrauterine transfer and fetal storage of vitamin D (45). By providing an injection of ^{3}H or ^{14}C labelled vitamin D^{3} to deficient rats prior to mating accumulation of the labelled vitamin D in the pup's tissue could be followed throughout their gestation and lactation periods (45). This was the first indication of how and where mammals transfer and store vitamin D in fetal tissue. It was concluded that primary transfer of vitamin D from the dams to the pups occurred during the third trimester of gestation while *in utero* and not after birth during the lactation period (45). This is the same period in which fetal mineralization occurs (46). The guinea pig model of vitamin D deficiency was used to investigate the effects of an *intra uterine* deficiency of vitamin D on fetal mineralisation (46). A deficiency at this stage of development led to low or undetectable $25(OH)D_3$ and $1,25(OH)_2D_3$ and a 10-15% lower body weight and whole body BMC by day 57 of gestation (46).

1.3 Vitamin D Nutrition in Women and Children

1.3.1 Vitamin D Status in Canada

Human studies show vitamin D deficiency exists in mothers and infants both in Canada (35,47-49) and worldwide (17,18,50-52). To highlight the severity of vitamin D deficiency in Canada, Rucker et al (53) reported that 34 % of women (27-89 years) living in Calgary had low vitamin D status in at least one season. Vieth et al (49) report on women

living in the Toronto region and observed the prevalence of vitamin D deficiency to be 14.8 % in white women and 25.6 % in non-white non-black women. During the winter months, the prevalence was not significantly affected by dietary or supplemental vitamin D intakes at values similar to the AI of 200 IU/d (5 μg/d) (14,49,54). Recently, Weiler et al. report that 46 % of healthy women are deficient in vitamin D immediately postpartum and that 35% of their newborn infants were deficient in vitamin D (35). All of these studies have been conducted at major Canadian cities that are located at a latitude greater than or equal to 43°N; for example Toronto is 43°N, Winnipeg ~49°N, and Calgary ~50°N. The high rate of deficiency is not surprising since cutaneous synthesis of vitamin D in these regions is thought to be limited to April through September (55). Thus many Canadians at risk for vitamin D deficiency and the consequence includes potentially limited bone mass or altered bone metabolism. Ward et al. (48) highlighted that not only is vitamin D deficiency an issue for infants, but that amongst Canadian children vitamin D deficient rickets is still present. An annual incidence rate of 2.9 cases of rickets per 100 000 children per year was documented between July 1, 2002 and June 30, 2004. The mean age of the children diagnosed with rickets was 1.4 years of age (48). Many of these infants were of First Nations status and were not receiving vitamin D supplements. For pregnant First Nations women vitamin D deficiency seems common, possibly due to the predominance of residence in more

extreme northern latitudes for those participating in existing reports, darker skin pigmentation and low dietary intake. Lebrun et al (56) reported that 76 % of Northern Manitoban women had 25(OH)D in the deficient range. Waiters et al (57) reported upon vitamin D status of women living in the Inuvik zone of the former Northwest Territories and vitamin D status. A predicted prevalence of deficiency was estimated at 48.4 % in First Nations mothers taking a vitamin D supplement and 88.6 % in those not consuming a supplement. For non-First Nations mothers taking a supplement the prediction was 15.1 % and without a supplement 63.5 %. Most maternal supplements contain 100 to 200 IU of vitamin D_3. Thus if the studies by Vieth et al (58) and Lebrun et al (56) Waiters et al (57) are reflective of the Canadian population of women of child bearing years, deficiency is likely very common, ranging from 15 % to 89 % of the population.

1.3.2 Vitamin D Status World Wide

Vitamin D deficiency is not only an issue in Canada, but worldwide. Summer born infants in Japan have higher vitamin D levels as measured by 25(OH)D status than winter born infants (47.3 ± 2.1 nmol/L vs. 22 ± 8.5 nmol/L) (59). Vitamin D is related to exposure to UVB radiation so the mothers of the summer born infants were either exposed to enough radiation to stimulate endogenous synthesis or had access to seasonally available vitamin D rich foods. It was found that 10% of breast-

fed infants living in Iowa were vitamin D deficient at 280 days of age (25(OH)D < 27.5 nmol/L). Vitamin D deficiency was greatly associated with winter, dark skin, and lack of supplementation. Of the breast-fed infants sampled in the winter the majority (78%) of them were vitamin D deficient (7).

Nehama et al (60) compared spring born infants to autumn born infants in Israel and it was concluded that spring born infants had a 46.5% lower 25 (OH)D status than those born in the autumn (28.3 ± 2.5 nmol/L vs. 45.3 ± 3.3 nmol/L). Namgung et al. found no difference in 25(OH)D status in relation to season in infants born in Ohio (summer: 60.5 ± 3.3 nmol/L vs. winter 54 ± 6.8 nmol/L) (61), however there was a difference noted in infants born in the summer versus the winter in Korea (30 ± 1.5 nmol/L vs. 10.8 ± 7.5) (62).

1.4 Assessment of Bone

To assess bone, many qualities need to be measured, some of which are not feasible to study in humans. Methods of evaluating bone include: bone densitometry, evaluation of biochemical markers of bone as well as biomechanical testing. Dual-energy x-ray absorptiometry (DXA) is the established standard for measuring BMD and is the most frequently used quantitative radiologic method to assess bone mass (12). It has been utilized successfully to measure BMC, BMD and bone area in adults, children and infants. It is most commonly performed on the spine, hip,

legs and arms since these are areas at risk of fracture (12). The advantage of DXA is that it is a quick and painless procedure which can be used to track losses and gains in BMD (12).

DXA has been validated for use in animal models including the guinea pig model, where both whole body and regional scans have been performed successfully (46,63). Although DXA is used as a substitute for testing bone fragility, biomechanical testing offers a clearer picture of the structural quality of the bone.

In basic animal studies biomechanical testing can be performed using a materials testing machine. Tests such as compression tests have been used to evaluate the ability of the spine to withstand a force and flexure tests (also known as three-point bending tests) have been used to determine the strength of long bones such as the tibia and femur. There are standards and recommendations to follow when conducting bone strength testing. Storage condition, storage and testing temperature, force and speed of the materials testing machine can alter the results (64-66). Some key terms in understanding biomechanics are stress which is the force per unit area or the force put on an area of bone, strain which is the percentage of change in length or relative deformation of the bone and the Young's modulus that is a measure of the elasticity (versus rigidity) of a bone in relationship to stress and strain (65). Other key terms include load which is the amount of force applied to the bone (67). Yield which is when the stress at which a marked increase in deformation occurs without an increase in load (67). A flexure test is when a specimen is supported on two beams and a load is applied to

the center (65). Stress and strain are calculated from incremental increases in load. Results are plotted on a stress strain curve where the maximum stress at failure is the flexure strength. Flexure extension is the term used to describe the amount the specimen moves during the application of the load during the flexure test (65).

Biochemical markers are effective tools used to assess bone formation or resorption. Markers of bone formation can be either direct or indirect products of osteoblast activity (68). Serum osteocalcin is directly related to osteoblast activity and is highest when bone formation is at its greatest such as during infancy (68). Human infants with low vitamin D levels also have low osteocalcin levels (69). Markers of bone resorption such as deoxypyridinoline (DPD) are degradation products of type I collagen cross-links in bone (68). Concentrations of DPD are expected to be low during periods of rapid bone growth (68). Young men born with low birth weight were found to have an increase in both osteocalcin and DPD (70). It was concluded that this biochemical combination represented accelerated bone turnover, but also a hormonal equilibrium in these young men (70).

1.5 Animal Models of Bone Development and Vitamin D Deficiency

1.5.1 Selecting the Appropriate Model

Animal studies present a reproducible ethically sound model in which to investigate the effects of vitamin D deficiency on bone growth, development and strength over both the short and long term. Finding the

appropriate model for the condition to be studied allows for a stronger translation of results to human health. With animal models, there is the opportunity to explore the effects of nutrient deficiencies as well as to provide an intervention dose of a particular substance. Animal models provide the advantage of studying the effects of a treatment in a shorter more controlled period of time compared to studies performed with human subjects. Some commonly used animal models in the study of bone growth and development are the mouse, rat, sheep and guinea pig (33,46,71,72). These models all have their advantages and disadvantages.

The animal model most frequently used in the study of bone mass and osteoporosis is the rodent. With the creation of knock out models, the mouse has become a valuable tool in the study vitamin D deficiency. Both the rat and mouse develop osteoporosis in response to dietary manipulation as well as ovariectomy. For example Karabelos et al. studied the effects of a single dose of 2000 IU of vitamin D_3 on bone mineralization in neonatal rats (26). The large bolus dose resulted in reduced whole body weight, BMC and BMD at 3.5 months of age compared to the vehicle injection when given to the rats within 24 hours after birth (33). This suggests that providing supplemental vitamin D in small daily doses may be the better strategy in which to recover and optimise vitamin D status while preventing the negative outcomes associated with the single large dose.

The rat model has been used to investigate the specific *intra*

uterine transfer and vitamin D storage in fetal tissue (45). Vitamin D deficient female rats were given an injection of ^{3}H or ^{14}C tagged vitamin D_3 before mating. Dams and pups were then culled at various stages during the pregnancy and lactation period in order to determine the main source of the pups' vitamin D stores. They concluded that the main supply of vitamin D for the pups was provided *in utero* by the dam. The number of vitamin D molecules in the pups rapidly increased during the last third of gestation going from 0.36 pmol/fetus on day 14 of gestation to 62.2 pmol/fetus at birth. This was the first article to indicate that in rodent mammals vitamin D is accumulated by the fetus during the end of the third trimester (45).

The effects of vitamin D deficiency on pregnant and lactating rats and their pups was also studied by Cancela et al. (71). Vitamin D deficient rats were mated and followed throughout pregnancy and lactation. Postpartum a supplement of 10 IU of vitamin D was given per day to the lactating rats. The 25(OH)D levels of the dams and pups in the both the deficient dams and pups was not detectable. Bone growth was suppressed in the vitamin D deficient animals and both the deficient mothers and pups exhibited severe signs of osteomalacia. Despite normalized 25(OH)D vitamin D status after maternal supplementation, it was found that although the vitamin D supplementation reversed the mineral, hormonal and skeletal abnormalities caused by the deficiency in the mother, these were not corrected in the pups by weaning age (71). Perhaps if the

supplement had been provided to the pups versus the mother, the pups may have had a better recovery.

As evident from the research presented above the rat is a popular animal to use in the study of bone mass and osteoporosis. This is because of its short gestation time, and its ability to have many offspring. This is advantageous for studying the effects on a treatment on mammals *in utero* and throughout development (68). Rats are also large enough to perform DXA at the end of their suckling period. However, while both the mouse and rat lend themselves nicely to long-term studies, measurement of vitamin D status at birth is limited due to small blood volume (68). Measurement of bone mass at birth is not feasible in the rat since the skeleton does not mineralize until the first week of life, unlike human fetuses which mineralize during the third trimester (60).

Maternal vitamin D deficiency has been shown to disrupt fetal growth and mineralization in humans (24), however development has been shown to be normal in fetal rats with vitamin D deficient mothers and vitamin D receptor gene-ablated mice (46). The use of rats and other small rodents has been criticized since they are chemically and functionally immature at birth and are born in large litters. Their greatest period of bone growth and formation occurs during the suckling and post-weaning period (46).

Unlike the rat, the guinea pig has a relatively long gestation period (65-72 days) after which their young are born chemically and physiologically mature. They also give birth to a small number of fetuses (on average 2-5) with the greatest period of bone growth and development occurring *in utero*. This starts

with ossification centers appearing in the clavicle, face and skull at day 26 of gestation and by day 33 of gestation development of the occipital and innominate bones (73).

In guinea pigs the changes measured in osteocalcin during pregnancy reflect those described in human pregnancies in which maternal osteocalcin remains low throughout pregnancy. Unlike humans and guinea pigs, osteocalcin concentrations in the rat peak at day 17 of neonatal life and even during fetal life the osteocalcin levels of the rat pup increase with increasing vitamin D levels (74). The whole body calcium content of the guinea pig at term is 11.31-14.29 g/kg fat free mass. This is only slightly higher than a human fetus which accumulates 9.55 g/kg free fat mass. Rodents such as the mouse and rat have much lower calcium content at the end of gestation (3.43 g/kg and 3.06 g/kg respectively) (74).

Models that are chemically and physiologically mature at birth provide the means in which to study the effects of nutrient deficiency *in utero* on fetal mineralization. Bovine and ovine models have been considered since like human fetuses, both have high serum osteocalcin at birth. They however have a different placental structure which affects the transfer mechanism of calcium and vitamin D due to the structural differences as well as the cost of maintaining such a model are draw backs (74). In ovine fetuses $1,25(OH)_2D$ both total and free are higher in the infant than the mother. Differences in trans placental passage and regulation of the calcium/phosphate and the calciotropic hormone (i.e. vitamin D) levels are higher in the fetus then in the mother. This is opposite to human pregnancies (74).

Ovine fetuses like the rat are born chemically and physiologically immature when compared to human infants. Due to the similarities in the placenta the guinea pig has become a popular model for transplacental calcium transport (74). The fat content of both fetal rats and sheep are extremely low (approximately 2%) whereas the fetal guinea pig's 11% fat content most closely resembles the human infants 16% body fat (72,75).

1.5.2 The Guinea Pig Model of Maternal Vitamin D Deficiency

Overall the guinea pig is a model that is much more suitable to study the effects of maternal vitamin D deficiency on bone as relevant to human health than the rodent ovine or bovine models. Data obtained from fetal guinea pigs in relation to bone markers and mineralization have been shown to be comparable with those of human fetuses (46,73-76). The guinea pig model has been validated as an appropriate model to study bone development and vitamin D deficiency (46,74). The maternal and fetal 1,25(OH) D_3 and osteocalcin levels respond in parallel to that of humans, with higher osteocalcin levels in the fetal guinea pigs than in the adults. Maternal and fetal vitamin D levels were comparable to levels found in humans and just like rats and humans fetal vitamin D is correlated to maternal vitamin D (fetal vitamin D being approximately 20% lower than maternal vitamin D) (74). This is because maternal transport through the placenta is the main source of fetal vitamin D.

As outlined in **Table 1-2**, the guinea pig is larger at birth and thus will permit assessment of vitamin D status and bone mass at birth and

thereafter. The fetal guinea pig also responds to vitamin D deficiency with low BMC and serum 25(OHD) like humans (40,46). This is exemplified in a study by Rummens et al. which reported that pregnant guinea pigs deprived of vitamin D produce offspring with low whole body bone mass measured as BMC (60). Deprivation of vitamin D beginning the first day after conception and continuing throughout pregnancy resulted in 25(OH)D concentrations <10 nmol/L compared to values well over 100 nmol/L in the control group. At c-section on day 56 of the typical 68 to 72 pregnancy, the vitamin D deficient fetuses had low to undetectable vitamin D as well as their body weight and BMC were 10-15 % lower then the control group (46). Unfortunately the offspring were not followed past delivery as they were prematurely born. To date, no study has examined if the intrauterine consequences of vitamin D deficiency lead to compromised bone mass by normal term delivery and thereafter in the guinea pig model.

Table 1-2: Comparison of Reproduction, Maturation and Bone Mass Characteristics of Humans, Rats and Guinea Pigs

	Human[1]	Sprague Dawley Rat[2]	Guinea Pig[3]
Length of gestation	38-42 wk	20-22 d	65-72 d
Typical #fetus/gestation	1	8-12	2-5
Evidence of fetal ossification	Yes	No	Yes
Size at birth	3.4 kg	5-8 g	75-95 g
Preferred nursing period	~ 6-12 mo	21 d	21 d
Whole body BMC at birth using DXA	74 g [20-22 g/kg body weight]	Not possible	1.5 g at gestation d 57 by c-section [~25 g/kg body weight][7]
Age at peak bone mass	25-39 y[4]	4-6 mo[5]	Not reported.
Age at which bone begins to decline	39+ y[4]	9-12 mo[6]	Not reported

[1] Information on birth weight and BMC from Weiler et al. (35).

[2]Information related to rat or guinea pig gestation and other general characteristics is from the Canadian Council on Animal Care (78) unless citation provided.

[3] Multi or tri-colored or non-inbred/out bread Hartley strains used vs other strains such as the inbred Hartley strain that develop osteoarthritis and are not appropriate for long-term study.

[4] Tenenhouse et al. (77).

[5] Peterson et al. (79).

[6] Wang et al. (80).

[7] Rummens et al. (46).

2.0 Chapter Two: Rationale

2.1 Rationale

Worldwide, vitamin D deficiency is reported in pregnant women and infants. The maternal diet has a powerful effect on the growth, and development of the fetus (23). It is unclear, however, if a vitamin D deficiency at a critical period of development, such as fetal life, permanently alters bone mass and metabolism, even after successful reversal of the deficiency. This is not easily studied in a prospective manner in humans since 40 years would be required to assess attainment of peak bone mass (12). The guinea pig is a unique animal model to address these questions since it mineralizes *in utero* as do humans and in contrast to the mouse or rat that mineralize bone postnatally. Additionally, the guinea pig is a proven model of dietary vitamin D deficiency with associated reductions in bone mass in 3rd trimester fetuses obtained by c-section (46). Recovery of bone mass and vitamin D status following vitamin D supplementation postpartum to weaning age has not been investigated using this model. This is the topic of this thesis.

2.2 Objectives and Hypotheses

The specific objectives of this research using the guinea pig model of maternal deprivation of vitamin D during pregnancy are to determine if:

1. Maternal dietary vitamin D deficiency throughout pregnancy alters bone mass in the offspring after spontaneous term birth;
2. Postnatal neonatal vitamin D supplementation corrects the vitamin D deficiency that was acquired *in utero* and does the supplement

alter bone mass by weaning age; and

3. Altered bone mass attributed to vitamin D deficiency is accompanied by altered bone strength at birth and weaning.

Hypotheses

It is hypothesized that offspring born to vitamin D deficient mothers will:

1. Have lower vitamin D status at birth and require post natal vitamin D supplementation to normalize vitamin D stores.
2. Have decreased bone mass and strength unless vitamin D supplementation is provided.

3.0 Chapter Three:

Postnatal Vitamin D Supplementation Normalized Neonatal Bone Following Maternal Dietary Vitamin D Deficiency in the Guinea Pig

3.0 Chapter Three

Postnatal Vitamin D Supplementation Normalized Neonatal Bone Following Maternal Dietary Vitamin D Deficiency in the Guinea Pig

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Running title: Consequences of intrauterine deficiency of vitamin D to bone mass at weaning.

3.1 Abstract

While vitamin D deficiency is common at birth, the consequences to growth and bone mass by weaning are unclear. This study was designed to determine if maternal dietary vitamin D deficiency in pregnancy has a negative impact on bone mass of full term neonates and if postnatal supplementation would recover the limited bone mass. Forty guinea pigs were randomized to receive a diet with or without vitamin D_3 during pregnancy. At birth, their offspring were randomized to receive 10 IU of vitamin D_3 or a placebo daily until d28. Measurements at birth and d28 included whole body and regional bone mass, osteocalcin and deoxypyridinoline, plus biomechanical testing of excised tibias and femurs. Main and interaction effects were detected using factorial ANOVA and post-hoc testing using Bonferroni tests. Offspring from deficient mothers had lower body weight, whole body and tibia bone mineral content (BMC) and lower osteocalcin and biomechanical integrity. By d28 this group had lower whole body bone density and femur BMC, unless supplemented. Interactions with gender showed males continued to have low 25(OH)D despite supplementation. In summary, postnatal vitamin D supplementation normalized neonatal bone following maternal dietary vitamin D deficiency, but does not lead to optimal vitamin D status in the guinea pig.

3.2 Introduction

The maternal diet has a powerful effect on the growth and development of the fetus (23). The vitamin D status of newborns has a direct correlation to maternal vitamin D status (74). In addition, vitamin D deficiency has been reported in many countries and cultures around the world in pregnant women and newborn infants (17,18,35,50-52). While vitamin D deficiency can be reversed in human infants through supplementation, it is unclear if deficiency *in utero* and early in infancy manifests as prolonged altered bone mass and metabolism. It has been suggested that the intrauterine environment may play a role in later bone health since low circulating levels of vitamin D during late pregnancy associate with lower whole body and lumbar spine bone mineral density (BMD) in children at the age of 9 years (40). The greatest amount of growth and development occur *in utero* and during the first year of life. Interestingly, vitamin D intake in the first year of life is also positively associated with higher bone mass in prepubertal girls (81). The difficulty in many of these human studies is that they are not controlled trials and thus the true effects of vitamin D status on bone are possibly confounded by other fetal and neonatal exposures.

Studies using animals as models of human systems present a reproducible controlled model in which to investigate the effects of deficiency on bone growth, development and strength. The animal models most frequently used in the study of bone mass are rodents. However, in both the mouse and rat measurement of vitamin D status at birth is limited due to small blood volume. Measurement of bone mass at birth is not feasible in rodents since the skeleton does not mineralize

until the first week of life, unlike human fetuses that mineralize during the third trimester (12,46,72). In one study, supplementation of vitamin D was provided to lactating rats who had been deprived of vitamin D throughout pregnancy with the goal of correcting vitamin D deficiency in pups. Even with supplemental vitamin D provided to the mother during lactation osteomalacia and secondary hyperparathyroidism developed in the pups 20d post partum (71). In another study using rats vitamin D was not required for placental transfer of calcium or phosphorus to the fetus, but becomes important to bone formation after birth (32).

The relevance of these rodent studies to fetal bone mineralization is questionable because of their large litters chemical and physiological immaturity at birth (73,74,76). Additionally, bone development is normal in fetal rats with vitamin D deficient mothers and vitamin D receptor gene-ablated mice (32) whereas in humans maternal vitamin D deficiency disrupts fetal growth and mineralization (24,35,46). The guinea pig is a non-rodent model validated to study neonatal bone development, bone mass and vitamin D status birth (46). The guinea pig is larger at birth than mice or rats and will thus permit assessment of vitamin D status and bone mass at birth and thereafter. Lastly, at birth, the guinea pig has a similar amount of mineral/kg body weight as does the human infant (72,75). Like human infants, the fetal guinea pig responds to vitamin D deficiency with reduced serum 25(OH)D and BMD (46,82), and is therefore hypothesised to require a vitamin D supplement postnatally.

Therefore, the objective of this study was through using the guinea pig model to determine if maternal dietary vitamin D deficiency in

pregnancy has a negative impact on fetal bone mineralization and if postnatal supplementation in offspring would recover the limited bone mass by weaning age.

3.3 Methods

Animal Husbandry

Prior to conduct of the study, ethical approval was obtained from the McGill University Animal Care Committee **(Appendix A)**. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (78).

Pigmented (28 day old) guinea pigs (40 female and 10 males; 4:1 breeding ratio) were purchased from Elm Hill Laboratories (Boston, Massachusetts, USA) to form the breeding colony. The room temperature was maintained at 21°C, with the lighting cycle from 0630 to 1830 h and was free of UVB exposure to prevent endogenous synthesis of vitamin D. At 3 months of age, guinea pigs were randomized to their treatment groups and then bred.

Upon presence of the vaginal plug, the sows were provided a vitamin D deficient (deficient group) or sufficient (control group) pelleted diet for the full gestation and lactation period and were given food and deionized water *ad libitum.* This approach was taken since it yields deficiency during the last trimester when fetal mineralization occurs (46). Sows were housed singly in cages with their offspring to 28 days

postpartum. At birth, the pups were randomized within litters to receive a vitamin D supplement (supplement group) or placebo (placebo group) beginning at birth and continuing to d28 of age (i.e. 1 pup per litter receiving either the vitamin D supplement or placebo). Any litters with more than 2 pups were culled to 2 per litter at either birth or d28, which was determined through randomization. Culled pups were used for bone strength testing. At 28 days of age, the pups were separated from the sow and housed in same sex pairs for a longitudinal study, to be reported elsewhere. Growth (body weight g and body length cm) was measured in pups at birth and d28.

Diet

All diets were in pellet form, isoenergetic, equal in minerals and with all nutrients except vitamin D at recommended amounts as per the National Research Council and purchased from TestDiet (TestDiet®, *Division of Land O'Lakes Purina Feed, LLC,* Richmond, IN, USA). The standard diet contains 1.2 IU of vitamin D_3/g diet and is made with identical ingredients and manufacturing as the vitamin D deficient diet with exception of addition of vitamin D. See **Table 3-1** for the diet composition. The diets were blinded and the sows were allocated to the test diet at random. The diet was stored at -4°C and food intake was monitored every two days.

Vitamin D Supplementation

At birth pups were randomized to receive either a supplement

containing 10 IU of vitamin D_3 or a placebo (control) of equal volume. Vitamin D supplements and placebo for offspring were provided in-kind by Euro-Pharm International (Montreal, QC, Canada) and were prepared via the same preparation method as human studies only in a lower vitamin D concentration (10 IU/ml versus 40 IU/ml). The dosage of 10 IU or a placebo was delivered by orally syringe daily until d28 of life. A daily supplement is preferred vs. one large bolus dosage at birth since a 0.05 mg (50 μg or 2000 IU) within 24 hr of birth in rats resulted in reduced whole body weight, bone mineral content (BMC) and bone mineral density BMD at 3.5 months of age compared to the vehicle injection (83). Diets and supplements were blinded using letter coding and only upon completion of measurements were the study groups un-blinded.

Blood Sampling

Blood was taken from the saphenous vein between 0800 and 1000 h for all samplings in sows or pups. In sows, sampling took place at conception then day 42 (beginning of 3rd trimester) of pregnancy and immediately postpartum for measurement of 25(OH)D only. Blood was sampled from pups within 48 h of birth and at d28. All samples were centrifuged at 3000 g for 30 min to obtain serum and plasma and stored at –80°C thereafter to ensure the biomarkers of bone metabolism were stable (84-86).

Table 3-1: Vitamin D Deficient Guinea Pig Diet Test Diet

Ingredients	%
Sucrose	29.4009
Corn Starch	21.8000
Soy Protein Isolate	16.7171
Powdered Cellulose	14.0000
Soybean Oil	6.0000
Casein - Vitamin Free	5.0000
Dicalcium Phosphate	2.6434
Potassium Citrate, Tribasic	1.1323
Monohydrate Calcium Carbonate	1.1263
Potassium Carbonate	0.6799
Salt	0.5908
Choline Chloride	0.2643
Ascorbic Acid	0.2335
Magnesium Oxide	0.1468
Vitamin and Mineral Premix	0.1468
Vitamin K Premix	0.0628
L-Methionine	0.0539
Ethoxyquin (a preservative)	0.0012

NOTE: the control diet (diet containing vitamin D) has 1.2 IU vitamin D_3/g diet.

Whole Body and Regional Bone Analysis

At birth and d28, the pups weight and body length from nose to anus were recorded. Bone mass was measured using dual-energy x-ray absorptiometry (DXA). Anaesthesia was induced using AErrane isoflurane gas (Baxter Inc., Mississauga, ON, Canada) at 5% in an induction chamber followed by maintenance at 2% delivered using a cone mask. Isoflurane gas has been successfully used in guinea pigs as anaesthesia prior to measurement of bone mass (87). The guinea pigs were measured prone with limbs extended and scanned in whole body and high-resolution modes using a Discovery Series 4500A densitometer (Hologic Inc., Bedford MA, USA), and the small animal software. Whole body, lumbar spine 1-4, femur and tibia were measured for bone area, BMC and BMD. Daily calibrations of the machine were performed using a spine phantom; the coefficient of variation with the phantom was 0.53% ± 0.01g/cm^2 for BMD, 0.66% ± 0.37g for BMC and 0.43% ± 0.23 cm^2 for area. DXA was also performed on the excised femurs of the culled animals in order to obtain BMC, BMD and area measurements ex vivo for comparison among groups with standardized positioning. Both right and left tibias and femurs were placed in the anterior to posterior direction and scanned separately. Bones were submersed in a water bath to imitate soft tissue. The coefficient of variation of three consecutive measurements was 5.3% for whole body BMC, 1.9% for spine BMC, 2.3% for tibia BMC and 0.9% for femoral BMC.

Tissue Sampling

Guinea pig litters were culled to two using randomization to necropsy at either birth or d28 after completion of the DXA. They were

anesthetized, which was induced and maintained at 5%. Blood was sampled using cardiac puncture. Tissues were removed, weighed and frozen in liquid nitrogen. The tissues were then stored at -80°C. Blood was centrifuged at 3000 g for 20 min, separated into serum and plasma and stored at -80°C until analysis. Both right and left tibias and femurs were excised, cleaned of adhering soft tissue, wrapped in saline soaked gauze and stored at -20°C until mechanical tests were performed.

Biochemistry

At birth and d28 serum biochemistry was measured in all offspring using samples obtained from the saphenous vein or cardiac puncture (if pups were culled). Serum 25(OH)D was measured using enzyme-immunoassay (ELISA) (Ref AC-57f1, Lot 59031, Immunodiagnostic Systems Limited, Boldon UK). This assay is specific for vitamin D_3 and also measures vitamin D_2, having 70% cross reactivity. The inter assay variation for the low and high controls was < 10%.

Osteocalcin concentration was measured to reflect osteoblast activity using ELISA (Ref 8002, Lot 903875, Quidel Corporation, San Diego CA). This assay has been validated for use in guinea pig samples (74). The interassay variation of the high and low controls was < 10% with a 1:40 dilution of serum used.

Deoxypyridinoline (DPD) concentration was measured to reflect osteoclast activity in serum using an ELISA (Ref 8032, Lot 903718, Quidel Corporation, San Diego CA). The interassay variation of the high

and low controls was < 10%. According to manufacturers specifications the antibody used in this assay cross-reacts with guinea pig samples.

Bone Strength

Bones stored at -20°C were thawed to room temperature (23°C) according to standard methods (64) and kept moist in saline soaked gauze (88). Right femur length, and the widths of the mid-diaphysis, knee, femoral head and neck as well as tibial length and mid-diaphysis width were measured using an electronic calliper (Control Company, Friendswood, Texas, USA) and the average of three measurements was taken. A test speed of 1 mm/min and a support span of 10 to 20 mm were employed with one loading point applying pressure to the bone. The fulcrum span was wider for the tibias than the femurs as well for the different time points but was maintained consistent across the samples within each bone type: birth femurs 10 mm, birth tibias 16 mm, d28 femurs 14 mm, and d28 tibias 20 mm. Load was applied at a constant displacement rate in the anterior-posterior direction, midway between the two fixed supports on which the bone was mounted. Bones were loaded to failure. Upon failure measurements were taken on the width of cortical defined as the distance from the exterior (periosteum) to the interior of the dense surface wall of the bone (endosteum) and cancellous bone defined as the canal width of the canal separating the opposite ends of the endosteum. These were measured using an electronic calliper (Control Company, Friendswood, Texas, USA) and the average of three

measurements was taken. Data was obtained on the load at yield, flexure load at yield, Young's Modulus, maximum flexure strain, maximum load, flexure load at maximum load flexure extension at maximum load, flexure strain at maximum load, flexure stress at maximum load and flexure stress at 50% of break (flexure extension).

3.4 Statistical Analysis

Data were analyzed for main effects of the gestational diets, postnatal supplementation, gender and time as well as interaction effects using factorial ANOVA (SAS Inst., Inc, Cary, NC. 9.1, 2003). Post-hoc testing was conducted for interaction effects using Bonferroni's multiple comparison test. Data were expressed as mean ± standard deviation was normally distributed. Differences were considered significant if $P<0.05$. Although gender and time were tested as a main effects they were only explored further when they interacted with maternal diet or postnatal supplement.

The estimated sample size required for this study was somewhat uncertain since no study has examined vitamin D status at birth and subsequent bone mass using the guinea pig. However, studies examining vitamin D deficiency *in utero* (46) or vitamin C (ascorbic acid) from weaning to aging (87) have used 8 to 10 per group. The primary outcome in this study was bone mass (at birth and weaning) assessed using BMC and BMD. It was assumed that a 10% reduction in bone mass is considered consistent with fracture and weak bones. Regional differences

have previously not been investigated using the guinea pig model. Precision of DXA is very good at 1-2% in small pups and <1% in larger rodents using the 4500A DXA (60). Therefore, the estimated size effect (10%) for reduced BMC for this study exceeds the error of measurement (1% maximum). The sample size used in this study (20 sows/diet group) with 2 pups per litter would yield 40 pups per maternal diet group at birth and 20 pups for each of the postnatal supplementation groups. A sample of greater than 20 per group was achieved and provided a power greater than beta=0.90 with alpha=0.01.

3.5 Results

Maternal Vitamin D Status

Thirty-eight of the forty sows were successfully mated and delivered healthy litters, as shown in **Figure 3-1.** Litter size was not different between maternal diet groups (control 2.6 ± 0.8 vs. deficient 2.9 ± 0.1, P=0.1815). One pup in the control group and two in the deficient group died at birth. In total 110 offspring were born and 104 were studied (**Figure 3-1**).

Two sows randomized to the vitamin D deficient group were removed from the study. One sow was removed due to complications during pregnancy and the other because of unsuccessful mating. There was no significant difference in the serum 25(OH)D of the sows before mating (at baseline), but, by day 42 of pregnancy sows in the deficient group had significantly lower 25(OH)D (**Figure 3-2**). This statistically significant difference remained throughout pregnancy to delivery as well as to the end of lactation at 28 d postpartum.

Neonatal Guinea Pigs

Vitamin D status of the pups at birth was indicated by 25(OH)D. Main effects were present for diet and time, 25(OH)D was significantly lower in the deficient group compared to control (21.0 ± 38.6 vs. 56.1 ± 27.5 nmol/L, P<0.0002). There was also a significant difference between vitamin D status of the pups at birth versus d28 (**Table 3-2**). Both male and female offspring from the deficient diet group plus placebo supplement had lower 25(OH)D at d28 compared to the male and female offspring from the control diet plus placebo and control diet plus supplement groups. Interaction effects between gender and

supplement were present; in the males, the 25(OH)D at d28 in the deficient diet group plus supplement was only significantly lower than control offspring (**Figure 3-3**).

Weight of the pups was lower in the vitamin D deficient group at birth (169.6 ± 97.9 vs. 155.3 ± 97.9 g, P=0.042), and d28 (279.1 ± 47.4 vs. 255.9 ± 44.2 g, P=0.024) (**Figure 3-4**). The postnatal supplement however did not alter weight or interact with time (P>0.05) and gender (P>0.05). There was no significant difference in the body length of the pups regardless of maternal diet or postnatal supplement (**Table 3-3**).

Bone Mass

Main effects of the maternal diet showed vitamin D deficient offspring had 6% lower whole body BMC (5.58 ± 2.69 g vs. 5.24 ± 2.52 g, P=0.012) (**Figure 3-5A**) regardless of time and gender as well as a 6.7% lower right tibia BMC (0.15 ± 0.07 vs. 0.14 ± 0.07 g, P=0.012) (**Figure 3-5B**) than the control group. As shown in **Table 3-4** there were no other significant differences amongst the groups. Interaction effects between maternal diet and postnatal supplementation showed that the deficient group was lower than the control group in whole body BMD (P=0.045) unless they received supplemental vitamin D. Right and left femur BMC was lower at d28 in the deficient males unless they received the supplement (P=0.012) (**Figure 3-6**) of the right femur BMC. No main effects or interactions amongst diet, supplement, time or gender were present between the 4 groups in the BMC, BMD or area of the spine (**Table 3-5**) *in vivo* or of the tibias (**Table 3-6**) or femurs (**Table 3-7**). There was also no

significant difference between the tibias and femurs when scanned *ex vivo* when compared at birth or d28 (data not shown).

Bone Biomarkers

There was a significant difference in serum osteocalcin concentration of the pups at birth ($P<0.01$) with greater values in the control group than the deficient group (70.3 ± 12.6 vs. 66.4 ± 7.0 nmol/L) (**Figure 3-7**). There were no statistically significant differences amongst the groups due to the placebo or supplement ($P=0.70$), or interaction between gestational diet and postnatal supplement ($P=0.095$). As expected there was a significant difference in the osteocalcin values at birth and d28. A main effect of time was present with serum DPD concentration being significantly different at birth than at d28 ($P=0.0075$). Vitamin D supplementation did not have an effect on the DPD concentrations by weaning **(Table 3-2).**

Mechanical Testing

There was no difference in the size and shape of the bones as determined through measurement of the femoral length, head, neck, mid-diaphysis, knee, and femur cortical and cancellous (data not shown). Tibia length, mid-diaphysis cortical and cancellous measurements were taken and none were found to be statistically significant (data not shown). No main effects of gestational diet or post-natal supplement were significant in the femur load at yield, flexure load at yield, maximum flexure strain, maximum load, flexure load at maximum load, and flexure extension at maximum load at birth and d28 (**Table 3-8 and 3-9**). Main effects of supplementation were present for the femur Young's modulus

between the supplement and placebo groups regardless of gestational diet **(Table 3-9).** Interaction effects showed flexure strain at maximum load and flexure stress at maximum load were significantly different amongst the treatment groups.

No main effects were found due to maternal diet or post-natal supplementation at birth or d28 in the tibia maximum flexure strain, flexure load at maximum load, flexure extension at maximum load flexure strain at maximum load and flexure stress at maximum load. Significant main effects due to the gestational diets were observed in the tibia load at yield zero, flexure load at yield zero, the Young's modulus, and the maximum load (**Table 3-10 and 3-11**).

Table 3-2: Serum 25-hydroxyvitamin D, osteocalcin and deoxypyridinoline concentrations of guinea pigs grouped by gestational diet and supplement received after birth at birth and day 28 of study.

Measurement	Time point	Control Diet		Deficient Diet		P-Values for Main Effect[1]			
		Placebo (n=24/24)[2]	Supplement (n=31/25)	Placebo (n=23/20)	Supplement (n=30/23)	Diet	Supplement	Sex	Time
25(OH)D (nmol/L)	Birth	56.8 ± 24.6	55.5 ± 9.9	16.4 ± 10.3	24.7 ± 50.1	0.0001[3]	0.10	0.12	0.0001
	Day 28	114.1 ± 60.5	118.9 ± 42.9	16.6 ± 20.1	47.8 ± 55.0				
Osteocalcin (nmol/L)	Birth	70.7 ± 14.0[a]	70.1 ± 11.3[a]	67.0 ± 8.4[b]	65.8 ± 5.2[b]	0.04	0.70	0.20	0.07
	Day 28	64.3 ± 10.3[A]	68.6 ± 9.8[A]	62.9 ± 7.9[A]	66.2 ± 9.2[A]				
DPD (nmol/L)	Birth	23.0 ± 15.3[A]	21.6 ± 4.1[A]	21.9 ±8.6[A]	23.2 ± 7.8[A]	0.49	0.66	0.59	0.0001
	Day 28	13.9 ± 10.3[B]	19.7 ± 7.6[A]	22.1 ± 8.9[A]	12.5 ± 8.9[B]				

Data are mean ± SD. DPD: deoxypyridinoline.

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups. Values with different upper case superscripts indicate differences between 2 time-points.

[2] Sample sizes are for birth followed by d28. Sample was reduced to accommodate tissue sampling.

[3] An interaction among diet, supplementation, gender and time (P= 0.045) was observed, thus post-hoc testing not shown here.

Table 3-3. Size of guinea pigs at birth and day 28 by study group.

Measurement	Time point	Control Diet		Deficient Diet		P-Values for Main Effect[1]			
		Placebo (n=24/24)[2]	Supplement (n=31/25)	Placebo (n=23/20)	Supplement (n=30/23)	Diet	Supplement	Sex	Time
Weight (g)	Birth	109.7 ± 2.1^{aA}	105.0 ± 19.1^{aA}	97.5 ± 17.7^{bA}	100.6 ± 18.6^{bA}	0.03	0.87	0.11	0.0001
	Day 28	319.9 ± 61.8^{aB}	312.3 ± 48.3^{aB}	293.9 ± 56.9^{bB}	304.2 ± 51.2^{bB}				
Length (cm)	Birth	15.4 ± 1.0^{A}	15.3 ± 0.9^{A}	14.7 ± 0.8^{A}	15.0 ± 1.1^{A}	0.20	0.42	0.21	0.0001
	Day 28	22.3 ± 1.5^{B}	22.4 ± 1.1^{B}	21.8 ± 1.2^{B}	22.3 ± 1.3^{B}				

Data are mean ± SD

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups. Values with different upper case superscripts indicate differences between 2 time-points.

[2] Sample sizes are for birth followed by d28. Sample was reduced to accommodate tissue sampling.

Table 3-4. Whole body bone mass of guinea pigs by gestational diet and supplement received after birth at birth and day 28 of study.

Measurement	Time point	Control Diet		Deficient Diet		P-Values for Main Effect[1]			
		Placebo (n=24/24)[2]	Supplement (n=31/25)	Placebo (n=23/20)	Supplement (n=30/23)	Diet	Supplement	Sex	Time
Bone Area (cm^2)	Birth	28.32 ± 4.19^{A}	27.49 ± 3.48^{A}	26.22 ± 2.85^{A}	26.98 ± 3.98^{A}	0.06	0.49	0.25	0.001
	Day 28	51.51 ± 6.65^{B}	51.04 ± 5.89^{B}	47.54 ± 5.89^{B}	49.84 ± 5.92^{B}				
BMC (g)	Birth	3.37 ± 0.67^{aA}	3.18 ± 0.58^{aA}	3.03 ± 0.49^{bA}	3.18 ± 0.63^{bA}	0.03	0.96	0.21	0.001
	Day 28	8.25 ± 1.55^{cB}	8.12 ± 1.27^{cB}	7.53 ± 1.39^{cB}	7.85 ± 1.33^{cB}				
BMD (g/cm^2)	Birth	0.12 ± 0.01^{A}	0.12 ± 0.01^{A}	0.12 ± 0.01^{A}	0.12 ± 0.01^{A}	0.34[3]	0.84	0.13	0.001
	Day 28	0.16 ± 0.01^{B}	0.16 ± 0.01^{B}	0.15 ± 0.01^{B}	0.16 ± 0.01^{B}				

Data are mean ± SD. BMC: bone mineral content; BMD: bone mineral density.

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups. Values with different upper case superscripts indicate differences between 2 time-points.

[2] Sample sizes are for birth followed by d28. Sample was reduced to accommodate tissue sampling.

[3] An interaction among diet and supplement, gender and time was observed (0.045).

Table 3-5. Spine bone mass of guinea pigs by gestational diet and supplement received after birth at birth and day 28 of study.

Measurement	Time point	Control		Deficient		P-Values for Main Effect[1]			
		Placebo (n=24/24)[2]	Supplement (n=31/25)	Placebo (n=23/20)	Supplement (n=30/23)	Diet	Supplement	Sex	Time
Bone Area (cm^2)	Birth	0.74 ± 0.11^{A}	0.75 ± 0.14^{A}	0.69 ± 0.07^{A}	0.70 ± 0.08^{A}	0.06	0.15	0.24	0.001
	Day 28	1.38 ± 0.13^{B}	1.42 ± 0.14^{B}	1.35 ± 0.13^{B}	1.38 ± 0.28^{B}				
BMC (g)	Birth	0.12 ± 0.03^{aA}	0.12 ± 0.03^{aA}	0.12 ± 0.02^{bA}	0.12 ± 0.02^{bA}	0.12	0.10	0.08	0.001
	Day 28	0.23 ± 0.04^{B}	0.24 ± 0.04^{B}	0.21 ± 0.04^{B}	0.27 ± 0.02^{B}				
BMD (g/cm^2)	Birth	0.16 ± 0.03	0.16 ± 0.2	0.17 ± 0.02	0.16 ± 0.02	0.50	0.92	0.36	0.57
	Day 28	0.17 ± 0.02	0.16 ± 0.02	0.16 ± 0.02	0.16 ± 0.01				

Data are mean ± SD. BMC: bone mineral content; BMD: bone mineral density.

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups. Values with different upper case superscripts indicate differences between 2 time-points.

[2] Sample sizes are for birth followed by d28. Sample was reduced to accommodate tissue sampling.

Table 3-6. Tibia bone mass of guinea pigs by gestational diet and supplement received after birth at birth and day 28 of study.

Measurement	Time point	Control		Deficient		P-Values for Main Effect[1]			
		Placebo (n=24/24)[2]	Supplement (n=31/25)	Placebo (n=23/20)	Supplement (n=30/23)	Diet	Supplement	Sex	Time
Right Tibia Area (cm^2)	Birth	0.88 ± 0.10^{A}	0.84 ± 0.13^{A}	0.80 ± 0.10^{A}	0.78 ± 0.10^{A}	0.07	0.45	0.06	0.001
	Day 28	1.3 ± 0.10^{B}	1.3 ± 0.10^{B}	1.30 ± 0.10^{B}	1.3 ± 0.20^{B}				
Right Tibia BMC (g)	Birth	0.80 ± 0.03^{aA}	0.08 ± 0.04^{aA}	0.07 ± 0.01^{bA}	0.07 ± 0.03^{bA}	0.02	0.26	0.06	0.001
	Day 28	0.19 ± 0.04^{B}	0.19 ± 0.05^{B}	0.17 ± 0.05^{B}	0.19 ± 0.04^{B}				
Right Tibia BMD(g/cm^2)	Birth	0.09 ± 0.02^{A}	0.09 ± 0.02^{A}	0.09 ± 0.02^{A}	0.11 ± 0.2^{A}	0.97	0.22	0.12	0.001
	Day 28	0.15 ± 0.02^{B}	0.14 ± 0.03^{B}	$0.1\ 3 \pm 0.03^{B}$	0.14 ± 0.02^{B}				
Left Tibia Area (cm^2)	Birth	0.09 ± 0.03^{A}	0.09 ± 0.02^{A}	0.08 ± 0.03^{A}	0.09 ± 0.02^{A}	0.09	0.92	0.81	0.001
	Day 28	1.33 ± 0.14^{B}	1.34 ± 0.13^{B}	1.30 ± 0.14^{B}	1.28 ± 0.17^{B}				
Left Tibia BMC (g)	Birth	0.09 ± 0.03^{aA}	0.09 ± 0.02^{aA}	0.08 ± 0.03^{bA}	0.09 ± 0.02^{bA}	0.01	0.39	0.07	0.001
	Day 28	0.22 ± 0.04^{B}	0.22 ± 0.04^{B}	0.20 ± 0.04^{B}	0.19 ± 0.04^{B}				
Left Tibia BMD(g/cm^2)	Birth	0.09 ± 0.02^{A}	0.19 ± 0.05^{A}	0.09 ± 0.02^{A}	0.09 ± 0.02^{A}	0.25	0.42	0.72	0.001
	Day 28	0.16 ± 0.02^{B}	0.16 ± 0.02^{B}	0.15 ± 0.02^{B}	0.15 ± 0.02^{B}				

Data are mean ± SD. BMC: bone mineral content; BMD: bone mineral density.

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups. Values with different upper case superscripts indicate differences between 2 time-points.

[2] Sample sizes are for birth followed by d28. Sample was reduced to accommodate tissue sampling.

Table 3-7. Femur bone mass of guinea pigs by gestational diet and supplement received after birth at birth and day 28 of study.

Measurement	Time point	Control		Deficient		P-Values for Main Effect[1]			
		Placebo (n=24/24)[2]	Supplement (n=31/25)	Placebo (n=23/20)	Supplement (n=30/23)	Diet	Supplement	Sex	Time
Right Femur Area (cm^2)	Birth	0.77 ± 0.08^{A}	0.73 ± 0.10^{A}	0.69 ± 0.08^{A}	0.70 ± 0.08^{A}	0.15	0.59	0.54	0.001
	Day 28	1.09 ± 0.12^{B}	1.09 ± 0.1^{B}	1.02 ± 0.14^{B}	1.07 ± 0.10^{B}				
Right Femur BMC (g)	Birth	0.09 ± 0.03^{A}	0.09 ± 0.03^{A}	0.08 ± 0.03^{A}	0.09 ± 0.03^{A}	0.24[3]	0.28	0.003	0.001
	Day 28	0.29 ± 0.06^{B}	0.29 ± 0.07^{B}	0.27 ± 0.08^{B}	0.30 ± 0.06^{B}				
Right Femur BMD(g/cm^2)	Birth	0.12 ± 0.03^{A}	0.12 ± 0.03^{A}	0.12 ± 0.04^{A}	0.13 ± 0.3^{A}	0.98	0.58	0.01	0.001
	Day 28	0.27 ± 0.06^{B}	0.27 ± 0.06^{B}	0.26 ± 0.05^{B}	0.24 ± 0.05^{B}				
Left Femur Area (cm^2)	Birth	0.76 ± 0.09^{A}	0.75 ± 0.09^{A}	0.70 ± 0.07^{A}	0.69 ± 0.14^{A}	0.06	0.12	0.33	0.001
	Day 28	1.08 ± 0.11^{B}	1.09 ± 0.10^{B}	0.97 ± 0.18^{B}	1.07 ± 0.10^{B}				
Left Femur BMC (g)	Birth	0.09 ± 0.03^{A}	0.09 ± 0.03^{A}	0.08 ± 0.03^{A}	0.09 ± 0.03^{A}	0.36[3]	0.58	0.02	0.001
	Day 28	0.32 ± 0.07^{B}	0.32 ± 0.05^{B}	0.28 ± 0.04^{B}	0.31 ± 0.06^{B}				
Left Femur BMD(g/cm^2)	Birth	0.14 ± 0.03^{A}	0.12 ± 0.03^{A}	0.13 ± 0.03^{A}	0.14 ± 0.04^{A}	0.53	0.52	0.40	0.001
	Day 28	0.29 ± 0.06^{B}	0.29 ± 0.04^{B}	0.28 ± 0.03^{B}	0.29 ± 0.04^{B}				

Data are mean ± SD. BMC: bone mineral content; BMD: bone mineral density.

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups. Values with different upper case superscripts indicate differences between 2 time-points.

[2] Sample sizes are for birth followed by d28. Sample was reduced to accommodate tissue sampling.

[3] An interaction among diet, supplementation, gender and time was observed (P=0.012).

Table 3-8. Biomechanical testing of right guinea pig femurs by gestational diet at birth.

Measurement	Control (n=6)	Deficient (n=6)	Main Effect Diet[1]
Load at yield (N)	-23.81 ± 5.67	-21.3 ± 4.58	0.62
Flexure load at yield (kgf)	2.28 ± 0.67	2.17 ± 0.47	0.68
Young's modulus (MPa)	659.13 ± 211.89	867.74 ± 278.14	0.39
Maximum flexure strain (mm/mm)	0.18 ± 0.04	0.24 ± 0.07	0.07
Maximum load (N)	-0.15 ± 0.03[a]	-0.38 ± 0.26[b]	0.02
Flexure load at maximum load (kgf)	0.02 ± 0.03	0.04 ± 0.03	0.16
Flexure extension at maximum load (mm)	0.43 ± 0.52	0.16 ± 0.48	0.76
Flexure strain at maximum load (mm/mm)	0.06 ± 0.08	0.02 ± 0.06	0.73
Flexure stress at maximum load (MPa)	0.13 ± 0.43	0.66 ± 0.48	0.11

Data are mean ± SD.

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups.

Table 3-9. Biomechanical testing of right guinea pig femurs by gestational diet and supplement received after birth at day 28 of study.

Measurement	Control Placebo (n=4)	Control Supplement (n=4)	Deficient Placebo (n=6)	Deficient Supplement (n=5)	Main Effect Supplement[1]
Load at yield (N)	-31.92 ± 21.17	-23.95 ± 26.87	-26.23 ± 21.30	-20.56 ± 27.12	0.45
Flexure load at yield (kgf)	3.26 ± 2.16	2.44 ± 2.74	2.67 ± 2.17	2.10 ± 2.77	0.45
Young's modulus (MPa)	665.70 ± 222.00	318.12 ± 175.94	536.73 ± 270.75	357.24 ± 170.70	0.09
Maximum flexure strain (mm/mm)	0.12 ± 0.02	0.17 ± 0.03	0.21 ± 0.08	0.16 ± 0.04	0.99
Maximum load (N)	-0.50 ± 0.45	-0.24 ± 0.01	-0.34 ± 0.27	-0.48 ± 0.36	0.67
Flexure load at maximum load (kgf)	0.05 ± 0.05	0.02 ± 0.01	0.03 ± 0.03	0.05 ± 0.04	0.67
Flexure extension at maximum load (mm)	-0.10 ± 0.11	-0.05 ± 0.08	-0.09 ± 0.07	0.29 ± 0.07	0.42
Flexure strain at maximum load (mm/mm)	-0.02 ± 0.02	-0.01 ± 0.01	0.044 ± 0.01	-0.18 ± 0.01	0.40
Flexure stress at maximum load (MPa)	0.57 ± 0.63	0.17 ± 0.05	0.38 ± 0.23	0.40 ± 0.21	0.45

Data are mean ± SD.

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups.

Table 3-10. Biomechanical testing of right guinea pig tibias by gestational diet at birth.

Measurement	Control (n=6)	Deficient (n=6)	Main Effect Diet[1]
Load at yield (N)	-24.39 ± 4.97 [a]	-16.81 ± 3.49[b]	0.007
Flexure load at yield (kgf)	2.48 ± 0.50 [a]	1.71 ± 0.36 [b]	0.01
Young's modulus (MPa)	1502.70 ± 467.76 [a]	957.99 ± 149.02[b]	0.0005
Maximum flexure strain (mm/mm)	0.17 ± 0.12	0.20 ± 0.16	0.95
Maximum load (N)	-0.67 ± 0.40	-0.46 ± 0.17	0.32
Flexure load at maximum load (kgf)	0.07 ± 0.04	0.05 ± 0.02	0.32
Flexure extension at maximum load (mm)	0.88 ± 1.07	1.20 ± 1.44	0.52
Flexure strain at maximum load (mm/mm)	0.12 ± 0.16	0.15 ± 0.19	0.62
Flexure stress at maximum load (MPa)	1.7 ± 1.14	1.43 ± 0.62	0.34

Data are mean ± SD.

[1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case superscripts indicate differences between the 2 diet groups.

Table 3-11. Biomechanical testing of right guinea pig tibias by gestational diet and supplement received after birth at day 28 of study.

Measurement	Control Placebo (n=4)	Control Supplement (n=4)	Deficient Placebo (n=6)	Deficient Supplement (n=5)	Main Effect Supplement[1]
Load at yield (N)	-43.11 ± 11.88	-48.42 ± 7.07	-38.61 ± 7.07	-36.19 ± 11.74	0.70
Flexure load at yield (kgf)	4.13 ± 1.38	4.93 ± 0.72	3.94 ± 0.72	3.69 ± 1.20	0.49
Young's modulus (MPa)	849.15 ± 22.47	982.00 ± 77.03	803.24 ± 177.35	660.20 ± 128.14	0.96
Maximum flexure strain (mm/mm)	0.27 ± 0.13	0.24 ± 0.06	0.26 ± 0.14	0.2 ± 0.09	0.45
Maximum load (N)	-0.82 ± 0.40	-0.80 ± 0.36	-0.71 ± 0.33	-0.40 ± 1.49	0.62
Flexure load at maximum load (kgf)	0.08 ± 0.04	0.08 ± 0.04	0.07 ± 0.03	0.04 ± 0.15	0.49
Flexure extension at maximum load (mm)	0.54 ± 1.09	-0.001 ± 0.001	0.29 ± 0.07	0.33 ± 0.07	0.49
Flexure strain at maximum load (mm/mm)	0.08 ± 0.2	-0.001 ± 0.01	0.06 ± 0.1	0.05 ± 0.0.1	0.96
Flexure stress at maximum load (MPa)	1.16 ± 0.45	1.10 ± 0.44	1.02 ± 0.44	0.40 ± 2.11	0.45

Data are mean ± SD. [1] P-values reflect main effects identified in a factorial ANOVA model. Values with different lower case

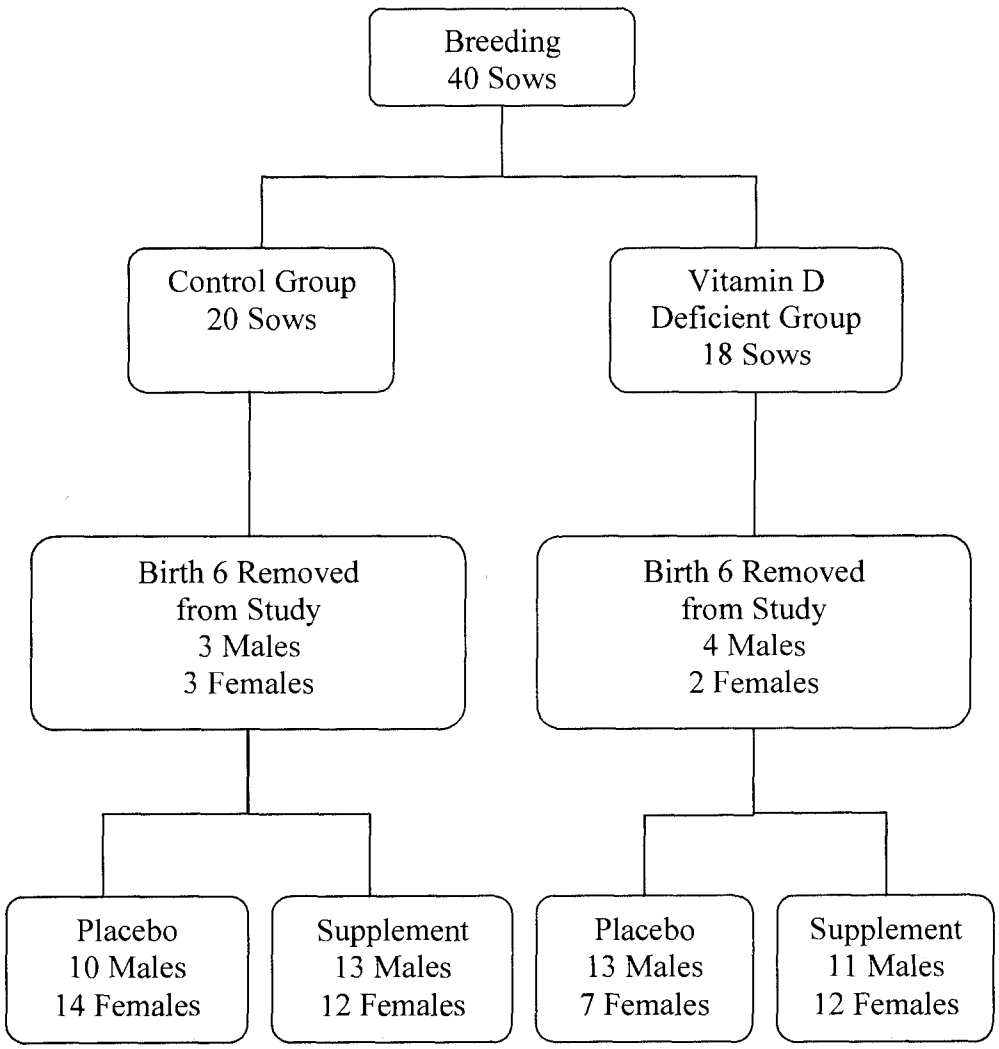

Figure 3-1. Study Design and Sample Size.

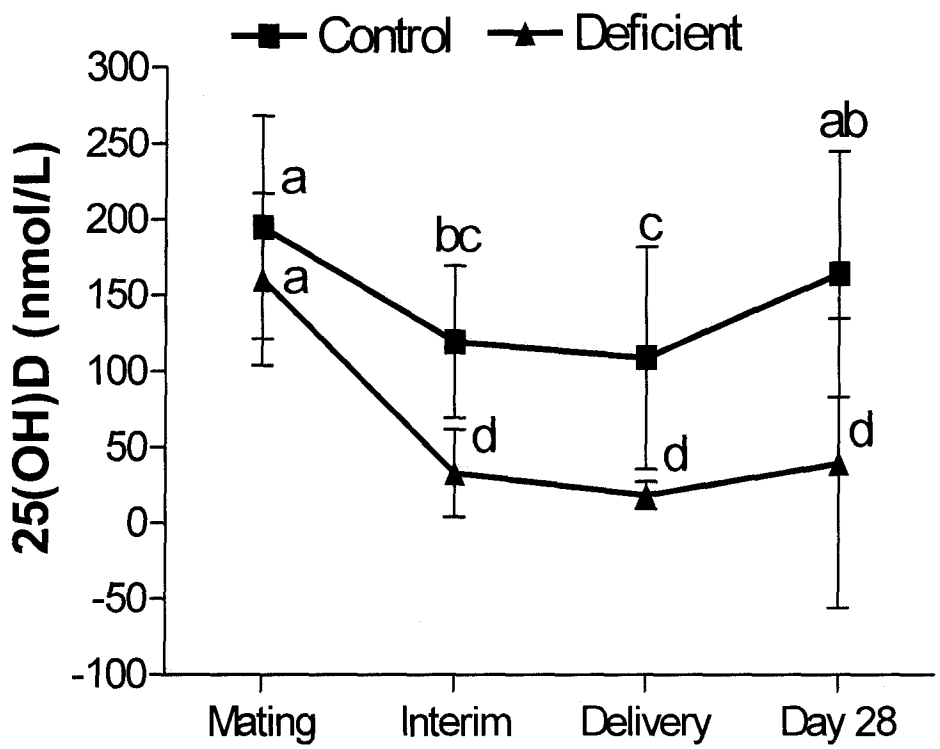

Figure 3-2. Sows serum 25(OH)D through pregnancy and lactation. Sample sizes were; control n=20, deficient n=18. Values with different superscripts indicate $P<0.05$ using post-hoc testing with Bonferroni's multiple comparison test. Interaction between diet and time ($P=0.034$) Data are mean ± SD.

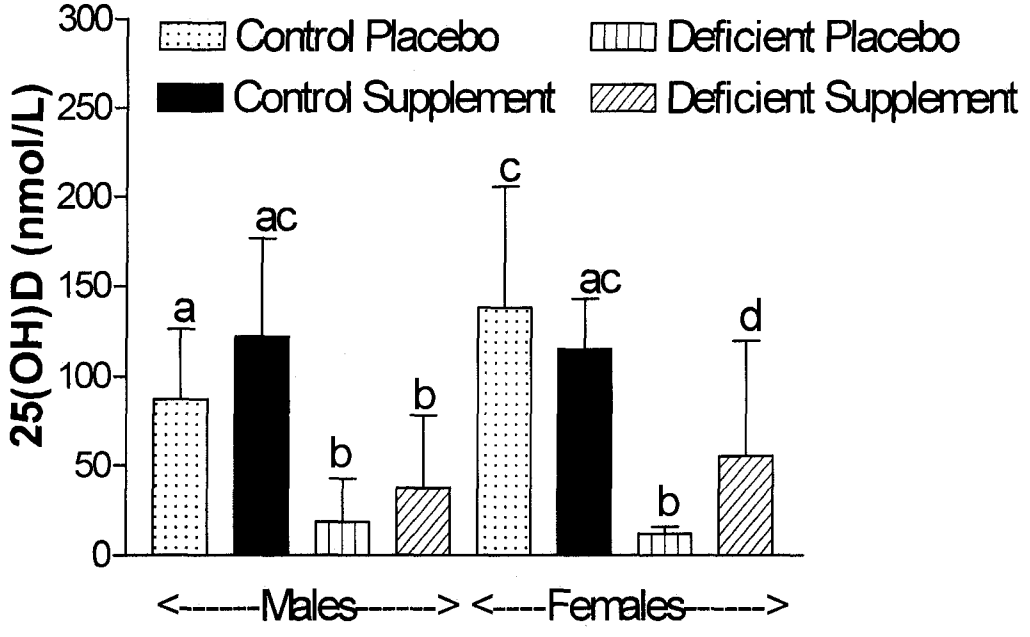

Figure 3-3. Serum 25(OH)D at d28 in male and female offspring. Sample sizes at day 28 were: control placebo male (n=10), control placebo female (n=14), control supplement males (n=13), control supplement females (n=12), deficient placebo male (n=13), deficient placebo female (n=7), deficient supplement male (n=11), deficient supplement female (n=12). Interaction between postnatal supplement, gender and time (P=0.0002). Values from post-hoc testing with different superscripts indicate $P<0.05$ using post-hoc testing with Bonferroni's multiple comparison test. Data at birth not shown. Data are mean ± SD.

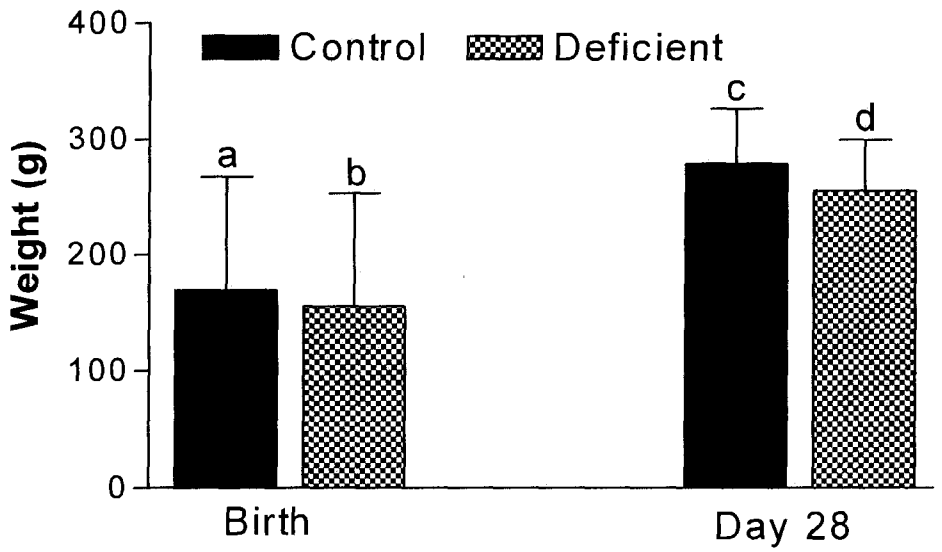

Figure 3-4. Body weight of offspring according to maternal diet during pregnancy and lactation. Sample sizes at birth and day 28 were: control (n=55,49), deficient (n=53,43). Main effect of maternal diet P=0.03. Values with different superscripts indicate P<0.05 using post-hoc testing with Bonferroni's multiple comparison test. Data are mean ± SD.

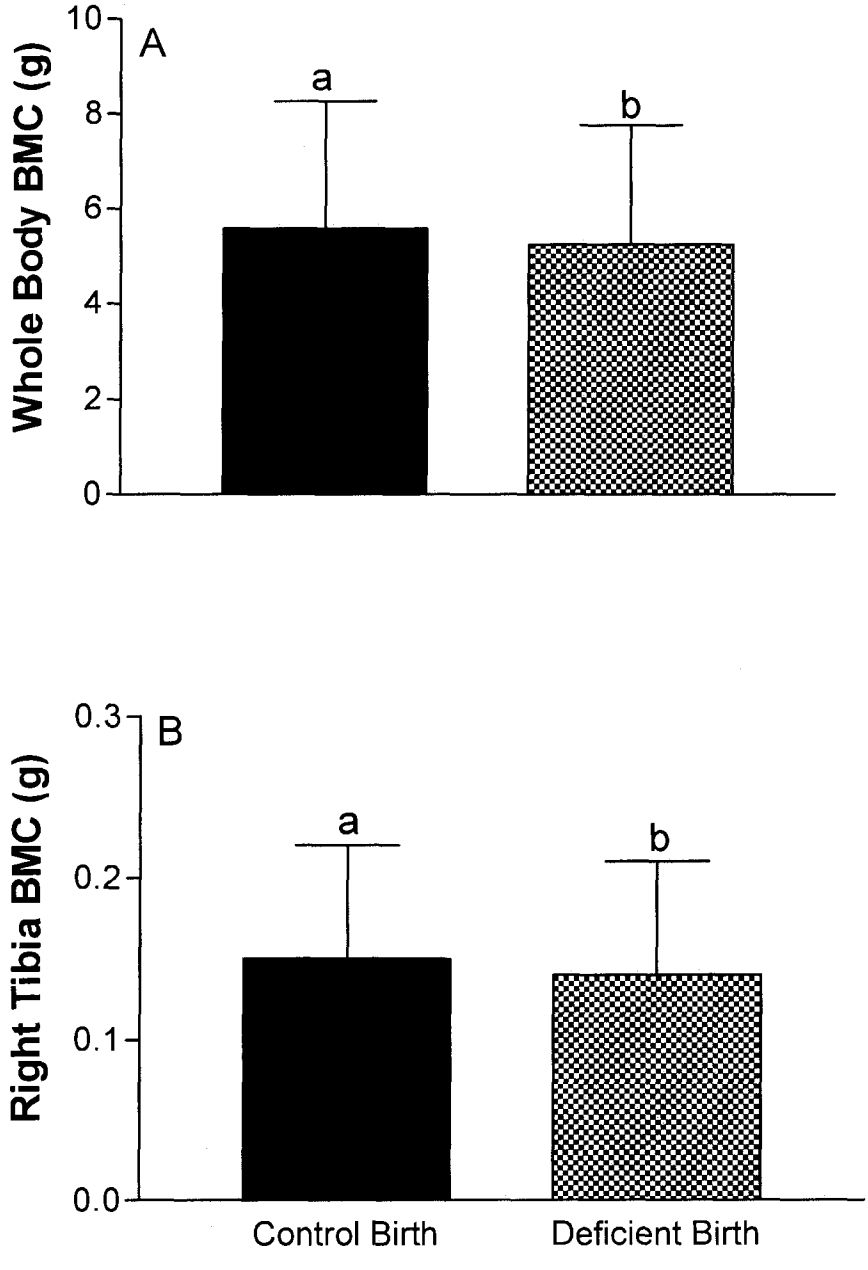

Figure 3-5A. Whole body (A) and right tibia (B) bone mineral content (BMC) at birth. In offspring from control (n=55) and deficient (n=53) groups. Main effects of gestational diet on whole body BMC (P=0.012) and right tibia (P=0.02). Values with different superscripts indicate P<0.05 using post-hoc testing with Bonferroni's multiple comparison test. Data are mean ± SD.

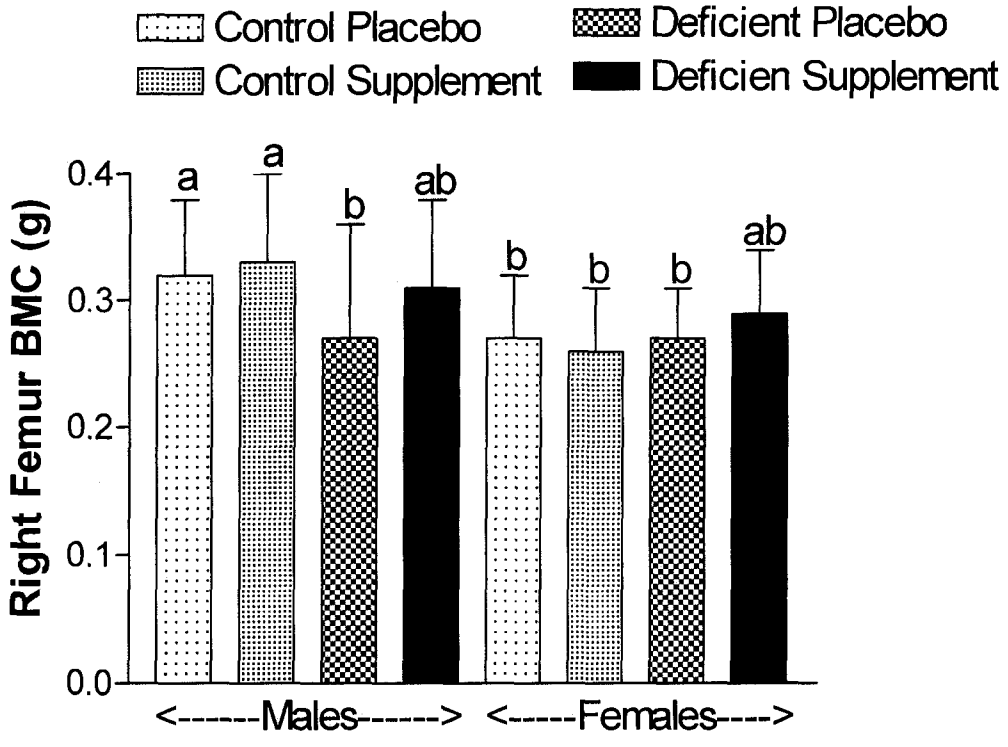

Figure 3-6. Right femur bone mineral content (BMC) of offspring at day 28 according to treatment and gender. Sample sizes day 28 was: control placebo male (n=10), control placebo female (n=14), control supplement males (n=13), control supplement females (n=12), deficient placebo male (n=13), deficient placebo female (n=7), deficient supplement male (n=11), deficient supplement female (n=12). Interaction between postnatal supplement and gender (P=0.012). Values from post-hoc testing with different superscripts indicate $P<0.05$ using post-hoc testing with Bonferroni's multiple comparison test. Data are mean ± SD.

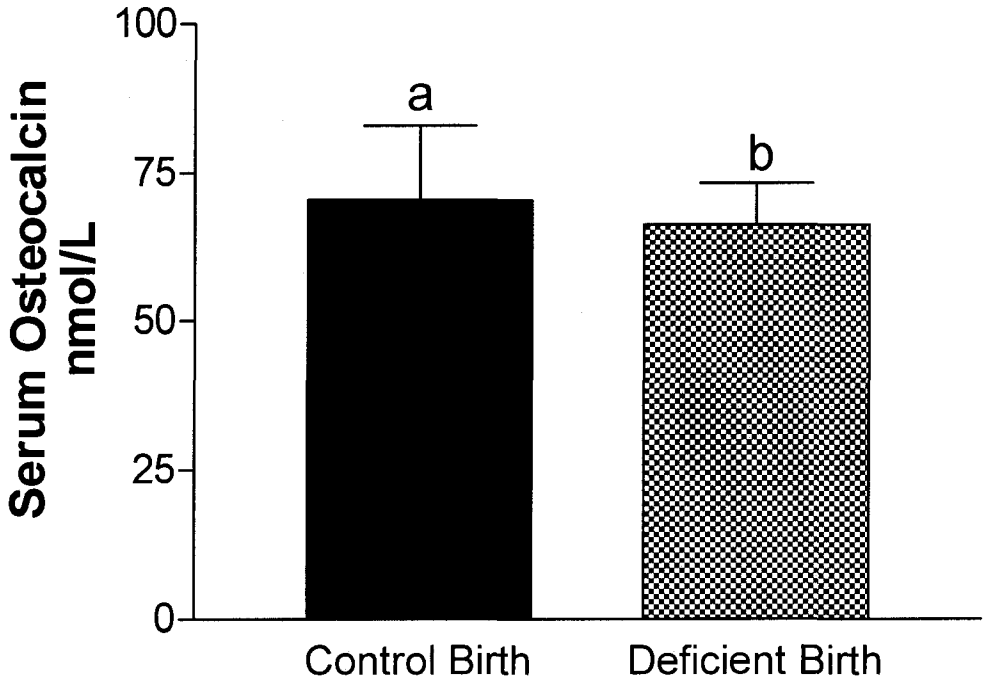

Figure 3-7. Serum osteocalcin in offspring at birth according to maternal diet, in offspring from control (n=55) and deficient (n=53) groups. Main effect of gestational diet (P=0.04). Values with different superscripts indicate P<0.05 using post-hoc testing with Bonferroni's multiple comparison test. Data are mean ± SD.

3.6 Discussion

While vitamin D deficiency can be reversed in human infants through supplementation (89), it is unclear if the long-term sequelae of deficiency *in utero* manifests as altered bone mass and metabolism. The objective of this study was to establish whether maternal dietary vitamin D deficiency in pregnancy has a negative impact on fetal mineralization and if postnatal supplementation in the offspring would normalize bone mass using a guinea pig model. The induction of dietary deficiency at mating yielded significant reductions in maternal serum 25(OH)D throughout pregnancy and resulted in offspring with vitamin D deficiency at birth defined as < 27.5 nmol/L (15). Thus the model was successful at inducing both maternal and neonatal vitamin D deficiency. The two hypotheses tested were, that offspring born to vitamin D deficient mothers will have lower vitamin D status at birth and require post natal vitamin D supplementation to normalize vitamin D stores. As well as have decreased bone mass and strength unless vitamin D supplementation is provided. These hypotheses were accepted based on the results presented above.

This is the first study to investigate the effect of vitamin D deficiency on bone mass of guinea pigs at full term birth and at weaning. Like the studies by Rummens et al. (46,74) and Verhaeghe et al. (46,74) that investigated maternal and fetal guinea pig vitamin D status at birth, this study also found the vitamin D concentrations of the pups were comparable to that of a human fetus (46,74). Vitamin D concentrations in the pups increased between birth and weaning, but males from the deficient group who received the postnatal supplement failed to

achieve normal concentrations, whereas the females in the same group reached values comparable to control. This suggests that females are able to mount a higher 25(OH)D than males in response to vitamin D supplementation. Alternatively males may require a larger dose of supplement in order to increase their vitamin D status. This is the first time the effectiveness of vitamin D supplementation and subsequent recovery have been investigated controlling for gender. None of the vitamin D deficient groups however reached the suggested optimal serum vitamin D levels over 75 nmol/L (90), whereas those in the control group on average had values over 75 nmol/L regardless of postnatal supplementation.

The low birth weight of the vitamin D deficient pups which was carried through weaning, suggests that a vitamin D deficiency may indirectly play a role in reduced bone mass. In humans, birth weight and weight at one year have been shown to be independent determinants of bone mass seven decades later (91). The differences in guinea pig weights at weaning age suggest that the *intra uterine* environment may have a far reaching effect on neonatal development, providing further evidence for the theory of fetal programming.

Maternal vitamin D deficiency reduces whole body BMC in offspring who were removed by laparotomy on day 57 of gestation (46). In the present study a maternal vitamin D deficiency reduced whole body BMC in offspring who were carried to term. This has also been noted in human infants, where relative to body weight whole body BMC was found to be lower in infants of vitamin D deficient mothers (35). The significance of the effect of maternal vitamin D deficiency was

demonstrated by Javaid et al, (40) who documented a lower maternal vitamin D status during late pregnancy was associated with lower whole body and lumbar spine BMC in the offspring at 9 years of age (40). Supplementation at birth has been shown to increase BMD at appendicular skeletal sites at 8 years of age (81). The guinea pig data also suggests that long bones are affected by an *intra uterine* deficiency of vitamin D. Both the tibia and femur demonstrated reduced bone mass. Since these bones are at risk of fracture later in life, optimization of mineral content in childhood is crucial to increase quality and longevity of life. Fortunately, interaction effects revealed that postnatal vitamin D supplementation normalized neonatal whole body BMD and femur BMC following maternal dietary vitamin D deficiency in the guinea pig.

Both osteocalcin and DPD were measured after birth and weaning. DPD has only been investigated in weanling male guinea pigs (92) and osteocalcin has been measured at day 63 of pregnancy in fetal guinea pigs (74,92). There were higher concentrations of osteocalcin in the pups who received the diet adequate in vitamin D. This suggests that there is a higher amount of bone formation in the pups that were vitamin D sufficient. This scenario also occurs in human infants with low 25(OH)D who have also been shown to have low osteocalcin levels (69). There was no difference in DPD values between the gestational diets suggesting that vitamin D deficiency in early life does not immediately accelerate bone resoption. This may be related to the postnatal period being a time of heightened bone turnover (12). The effects of an *intra uterine* vitamin D deficiency may be realised later in life once growth is complete.

Biomechanical testing revealed that increased tibia bone strength was due to the maternal diet, which provided adequate vitamin D, with an increase in energy required to break the tibia through an increase in the Young's modulus, maximum load and the flexure load. The effect of supplementation on femoral Young's modulus was not expected with there being a higher Young's modulus in the pups who had received the placebo supplement (both deficient and control groups) over the two groups who received post natal supplementation. This could have been due to reduced mineralization in the bones of the non-supplemented group making it more flexible and more willing to bend before breaking. However, due to the small sample size available for biomechanical testing this could not be further investigated. There was also an increase in the amount of stress that could be put on the femurs of the deficient group at birth before failure; this too might have been related to lower mineral content. The elastic nature of bone in vitamin D deficiency manifests as bowed legs in human infants, thus the guinea pig model appears to be very reflective of the human scenario.

There are many strengths of this novel study such as the large sample size, as well as biochemical and biomechanical analysis to the support the DXA analysis. The primary outcome in this study was bone mass (at birth and weaning) assessed using BMC and BMD. A sample size of greater than 20 per group was achieved and provided a power greater than beta=0.90 with alpha=0.01. This data was supported through biochemical analysis of bone markers and serum 25(OH)D measurements. In addition serum 25(OH)D concentrations were measured in the sows to confirm deficiency was present by

the third trimester of pregnancy when mineralization of bone takes place in the fetus. The DXA data was also supported by bone strength testing, this was important in determining if a decrease in BMC or BMD leads to reduced bone strength and subsequent fragility and risk of fracture.

In human infants the cause of vitamin D deficiency is not always clear and can be present in association with other dietary deficiencies that may affect bone. Furthermore, the onset of deficiency during pregnancy is almost never known. While vitamin D deficiency can be reversed in human infants through supplementation, it is unclear if the consequences of deficiency *in utero* manifests as altered bone mass and metabolism at weaning age. Thus this thesis data more clearly suggests that postnatal vitamin D supplementation normalizes neonatal bone following maternal dietary vitamin D deficiency in the guinea pig. This information can be used to drive vitamin D intervention strategies in human pregnancy and infancy with eventual implications for public policy on vitamin D fortification and supplementation practices.

Future studies are required to determine the optimal dose of vitamin D. Whether there are gender differences in the dosage and dosing schedule required for optimal bone development of males and females. Long-term studies are required to determine the consequences of intrauterine deficiency of vitamin D to bone mass across the lifespan. Also the role and requirement for vitamin D in pre-term versus full-term gestational infants will have to be examined.

3.7 Acknowledgements

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4.0 Chapter Four: General Discussion

4.0 General Discussion

This is the first study to determine if a vitamin D deficiency created *in utero* will have postnatal effects and if they can be corrected through vitamin D supplementation after birth. It was shown that a maternal diet deficient in vitamin D produced offspring who had lower whole body BMC and tibia BMC than the offspring of sows who were provided adequate vitamin D. Unless they received the supplemental vitamin D the deficient group was lower than the control group in whole body BMD and femur BMC. This data was supported through biochemical analysis of bone markers and serum vitamin D measurements.

Vitamin D deficiency leads to decreased absorption of calcium from the small intestine (12). With reduced dietary calcium absorption bone calcium is dissolved in order to maintain adequate blood calcium levels for metabolic, signal transduction and neuromuscular activities (12). This mechanism may have lead to the reduced BMC in the vitamin D deficient pups.

Growth supports an increase in vitamin D due to the increased requirement of calcium for bone development (12). The vitamin D deficient offspring weighted less that the offspring who received vitamin D suggesting that vitamin D may play a roll in subsequent growth. This may also have an effect on subsequent bone health since adult women born small size for gestational age have higher circulating $1,25(OH)_2D$ (24).

This model of vitamin D deficiency also yielded lower osteocalcin levels as is observed in human infants (74). The DXA data was also supported by bone strength testing using a materials testing machine. This was important in determining if a decrease in BMC or BMD leads to reduced bone strength and subsequent fragility and risk of fracture. Our sample size was adequate to detect differences in DXA measurements and biochemical tests amongst groups and genders. However our small sample size form the biomechanical tests may have limited our ability to fully assess the effect of maternal diet and postnatal supplementation on bone strength . The strengths of this study are that this is the first time that the effects of an intrauterine deficiency of vitamin D have been studied in full term pregnant guinea pigs who like humans mineralize *in utero*. Serum vitamin D concentrations confirmed that vitamin D deficiency was present by the third trimester of pregnancy when mineralization of bone takes place in guinea pigs. Further, in humans the maternal fetal transfer of 25(OH)D seems to be regulated in that maternal values are higher than those of newborn offspring (35). The pups' vitamin D status was much lower than maternal vitamin D status further confirming that this is an appropriate model to study maternal vitamin D deficiency and the effects on fetal and newborn nutrition. It suggests that the maternal diet affects the status of their off spring. The mechanism of this is unclear and whether it is through placental regulation or another mechanism will need to be investigated. Maternal vitamin D status increased between delivery and

d28 this could be due to the mobilisation of fat stores which occurs during lactation (93).

Another strength, yet potential limitation of the study, is that this is the first study to examine the effects of vitamin D deficiency and subsequent recovery of bone using the guinea pig model. Therefore the growth rate and development of the guinea pig especially in relation to bone were uncertain. As well, the amount of vitamin D in the guinea pig milk has yet to be determined and would have been advantageous to further validate the model as representative of the human scenario; i.e. low maternal milk vitamin D. Lastly, it would also have been advantageous to measure parathyroid hormone (PTH) because of its role in indirectly stimulating production of the active form of vitamin D in the kidney along with direct effects on bone metabolism. PTH and 25(OH)D levels are inversely related and often used to target optimal vitamin D status. There are currently no available assays for parathyroid PTH in the guinea pig. This is due in part to the fact that many assays are based on guinea pig antibodies to rat or human PTH and there is low demand for the product.

The diets used in this study have been used successfully by others (46) and there is enough information to demonstrate that the guinea pig mimics the human *intra uterine* response to vitamin D deficiency that manifests as reduced bone mass. Delivery of the supplement was very feasible since guinea pig pups can be easily hand reared from birth and due to their chemical and physiological maturity supplementation was

easy to administer (75). The delivery was given orally as would be the case for human infants and the vehicle itself was the matrix from a human infant vitamin D supplement. The product itself was also manufactured, specifically for this study, by a company that manufactures infant supplements. Thus the diets and supplements were major strengths of the study.

In retrospect a higher dosage of supplement could have resulted in stronger results for the male offspring. However, to have bone recovery after weaning with only a 10 IU supplement of vitamin D in the females, demonstrates the importance of vitamin D in bone health as well as the potential to reverse the negative affects manifested *in utero*. This has profound implications for health care and policy making suggesting that a mothers nutrient deficiency does not signify that her offspring has to suffer the potentially life threatening consequences associated with vitamin D deficiency.

Although the supplement was adequate for maintenance of vitamin D status and bone recovery in the pups it was not adequate to optimize vitamin D status in the deficient group regardless of gender. Conversely, a higher dosage supplement would not be required if maternal vitamin D status had been optimal. In fact a lower dose supplement may have maintained optimal vitamin D since values in the control placebo group increased from birth to d28. In that case the postnatal supplement would be important in maintaining vitamin D status

rather than correcting a vitamin D deficiency. This is the ideal case since maintenance of vitamin D status ensures optimal bone growth both *in utero* and after birth, where as postnatal correction of the deficiency means that there is a crucial period of time in which bone growth may not be optimised.

The lower vitamin D status in the males who were deficient at birth and subsequently received vitamin D supplementation could be related to an increased need for production of muscle (93). Clements et al. (45) discovered that vitamin D was mostly stored in the muscle tissue of fetal rats. Although no difference was found in body composition between males and female offspring between birth and d28 vitamin D may have been stored for subsequent growth and development.

In summary although the role of vitamin D in bone health and the prevention of rickets has been known since the late 1930's (12), and that vitamin D deficiency can be reversed in human infants through supplementation (35), it was unclear if the consequences of deficiency *in utero* manifest as altered bone mass and metabolism at weaning age. This thesis data suggests that postnatal vitamin D supplementation normalized neonatal bone following maternal dietary vitamin D deficiency in the guinea pig.

Future studies are required to determine the optimal dose of vitamin D, and to investigate whether the deficiency *in utero* programmes for low peak bone mass. Whether there are gender differences in the dosage and

dosing schedule required for optimal bone development of males and females also requires investigation. Long term studies are required to determine the consequences of intrauterine deficiency of vitamin D to bone mass across the lifespan. As well, biomechanical testing in areas such as the lumbar spine, ulna and regions of the femur such as the femoral head should be examined. These are areas with areas with increased risk of fracture with age (12).

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